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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/56, G01N 33/86

A1

(11) International Publication Number: WO 99/47699

(43) International Publication Date: 23 September 1999 (23.09.99)

(21) International Application Number: PCT/EP99/01599

(22) International Filing Date: 11 March 1999 (11.03.99)

(30) Priority Data:
98105043.8 19 March 1998 (19.03.98) EP

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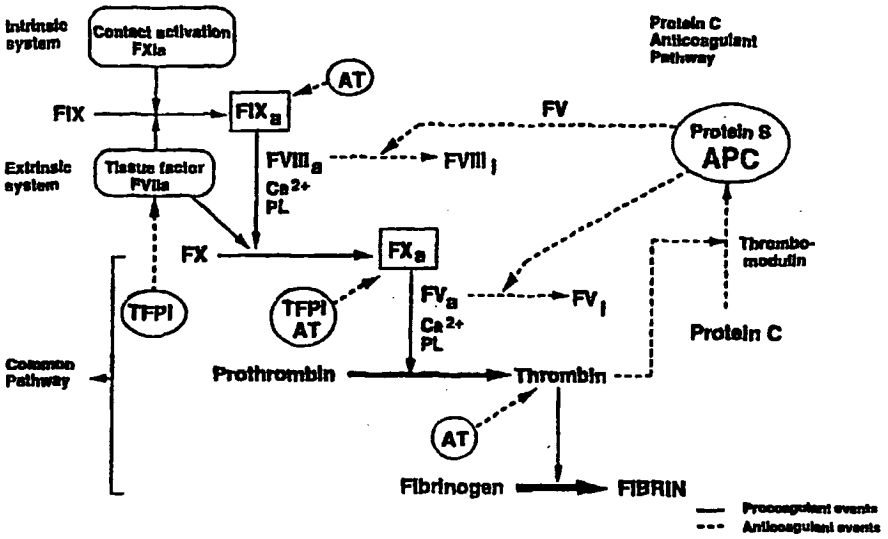
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SCREENING FOR BLOOD COAGULATION DEFECTS USING METAL IONS



(57) Abstract

In vitro photometric method for qualitative screening and quantitative determination of the functional activity of components of the Protein C anticoagulant pathway of blood coagulation, comprising measuring the conversion rate of an exogenous substrate by an enzyme, the activity of which is related to the Protein C anticoagulant activity, in a blood sample of a human comprising coagulation factors and said exogenous substrate after at least partial activation of coagulation through the intrinsic, extrinsic or common pathway and triggering coagulation by adding calcium ions; and comparing said conversion rate with the conversion rate of a normal human blood sample determined in the same way, comprising adding further metal(s) ions to said sample; kits and reagents for use in said method.

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SCREENING FOR BLOOD COAGULATION DEFECTS USING METAL IONS

Description

Field of the Invention

The present invention relates to in vitro photometric methods, kits and reagents for qualitative screening and quantitative determination of the functional activity of components of the Protein C anticoagulant pathway of blood coagulation, comprising measuring the conversion rate of an exogenous substrate by an enzyme, the activity of which is related to the Protein C anticoagulant activity, in a blood sample of a human comprising coagulation factors and said exogenous substrate after at least partial activation of coagulation through the intrinsic, extrinsic or common pathway and triggering coagulation by adding calcium ions; and comparing said conversion rate with the conversion rate of a normal human blood sample determined in the same way.

Background of the Invention

Maintenance of a proper hemostasis is the result of a careful balance between pro- and anticoagulant activities. After a trauma, coagulation is triggered primarily through activation of coagulation Factors IX and X (FIX, FX) by tissue factor (also denoted tissue thromboplastin) and Factor VII

(FVII) followed by generation of thrombin, which in turn cleaves fibrinogen to form soluble fibrin. After crosslinking by Factor XIII, a three-dimensional insoluble gel clot is obtained which prevents further blood losses.

Regulation of this highly potent system - shown schematically in Figure 1 of the drawings - is accomplished by a balanced relation between procoagulant events (shown with solid line arrows) and anticoagulant events (shown with dashed line arrows). The anticoagulant events comprise 1) inhibition of already formed thrombin by antithrombin (AT) and α_2-macroglobulin and 2) prevention of further thrombin formation by the Protein C anticoagulant pathway. Here, activated Protein C (APC) inactivates the coagulation proteins Factor VIII and Factor V in their activated forms (FVIIIa, FVa) through proteolytic cleavage. In addition inhibition of generated Factor Xa is also accomplished by antithrombin and tissue factor pathway inhibitor (TFPI), the latter also inhibiting the tissue factor/Factor VIIa complex. Factor VIIIa and Factor Va act as cofactors in the activation of Factor X and prothrombin, respectively, and increase the reaction rates of these processes about 1000-fold each. Thus, these cofactors act as potent stimulators of the coagulation. Their inactivation by APC therefore essentially stops further thrombin generation, thus providing a strong anticoagulant effect. Protein S and also Factor V act as cofactors to activated Protein C (APC).

As shown in Figure 1, the activation of coagulation through the intrinsic or extrinsic systems results in the activation of Factor X, a key component in the final common pathway. In the intrinsic system, the initial event is the activation of contact factors (Factor XII, prekallikrein) followed by the activation of Factor XI, which in turn activates Factor IX. In the extrinsic system, Factor IX and Factor X are activated by the tissue factor/Factor VIIa complex.

As Figure 1 as well shows, calcium ions have to be present in several of these reactions. The activation of Factor X by Factor IXa and of prothrombin by Factor Xa requires procoagulant phospholipids. In vivo, this is provided

by the membrane surface of activated platelets; in vitro by platelet extracts, purified phospolipids, synthetic phospolipids and/or crude phospholipid extracts from suitable sources. The total and free calcium ion concentration in native plasma is about 2.4 and and 1.2 mmol/L, respectively. Typically, calcium ion concentrations used in analytical methods for determination of coagulation or anticoagulation factors are in the range 1.5 -10 mmol/L. The concentrations of other metal ions in plasma are lower, typical values for total concentration being 1 mmol/L for Mg^{2+} and 5 - 40 µmol/L for Zn^{2+}, Cu^{2+} and Mn^{2+}.

Defects in the Protein C anticoagulant pathway may cause a risk of thrombosis due to a decreased capacity to prevent thrombin formation. Such defects may be due to deficiencies in the activity of Protein C and/or its cofactor Protein S. Another recently detected defect is a point mutation in the Factor V gene (G→ A) at nucleotide 1691, resulting in the amino acid substitution Arg (R) → Gln (Q) at position 506 in Factor V/Factor Va, denoted FV:Q^{506} or Factor V Leiden. Heterozygosity and homozygosity for this mutation are often denoted FV:R506Q and FV:Q506Q, respectively. This mutation is at one of the three APC cleavage sites (amino acids 306, 506, 679) in Factor Va, which confers an impairment of its degradation by activated Protein C (APC), denoted APC resistance.

APC resistance is to be considered a blood coagulation disorder recognized by an abnormally low anticoagulant response to activated Protein C (APC) and the determination of said APC resistance may be used to screen for and diagnose thromboembolic diseases, such as hereditary thrombophilia or for determining the risk for a human to acquire a manifestation of this blood coagulation disorder (Dahlbäck B, *EP-608 235*).

Hence, there is a need to investigate these components of the Protein C anticoagulant pathway in the evaluation of thrombotic patients and potentially also to screen for abnormalities of Protein C, Protein S and Factor V anticoagulant activity in situations connected with an increased risk of thrombosis such as before surgery, during trauma and during pregnancy or

in connection with the use of oral contraceptive pills or hormone replacement therapy. Currently, clotting and/or chromogenic assays are available for analysis of Protein C and Protein S activity as well as for the detection of APC resistance, which to at least 90% is due to the Factor V mutation at position 506.

Protein C activity is typically measured after activation of the endogenous Protein C, contained in a plasma sample, by thrombin or by a snake venom enzyme from Agkistrodon contortrix contortrix (Stocker K, *EP 203 509*), commercially available as the reagent Protac®C (Pentapharm AG, Basel, Switzerland). The concentration of Protac®C in the activation mixture is typically about 0.1 U/mL or above since otherwise an insufficient activation of Protein C may be obtained (Martinoli J, Stocker K.*Thromb Res* 43, 253-264 (1986); Mc Call F et al.*Thromb Res* 45, 681-685 (1987)).

After activation by Protac®C, the protein C activity is determined with a clotting or chromogenic assay (Bertina R. *Res Clin Lab*, 20, 127-138 (1990); Marlar R, Adcock DM. *Hum Pathol* 20, 1040-1047 (1989), Rosén S, EP 486 515). In clotting methods, coagulation is triggered through the intrinsic pathway by using APTT reagents or through the extrinsic pathway with the use of tissue factor. In both cases calcium ions are added to a final concentration of usually 5-10 mmol/L. Commercial kits and reagents are available for determination of Protein C activity such as Acticlot™ C (American Diagnostica), Stachrom Protein C (Diagnostica Stago), Staclot Protein C (Diagnostica Stago), Coamatic® Protein C (Chromogenix AB) and Protein C Activator (Behring Diagnostica).

The activation of Protein C by thrombin is stimulated about 1000-fold by thrombomodulin, an endothelial cell membrane protein (Esmon CT and Owen WG. *Proc Natl Acad Sci* 78, 2249-2252 (1981)). The use of thrombin/thrombomodulin as activator of Protein C for analysis of Protein C and/or Protein S activity in plasma samples, utilizing a photometric method, is also known (Pittet J-L and Aiach M, *FR Pat Appl. 2689 640-A1*).

Protein S activity is determined from its stimulation of the activity of

APC in its degradation of Factor Va and/or Factor VIIIa. Typically, in such assays a standardized amount of APC is added to a plasma sample or activation of endogenous protein C is performed whereafter the clotting time is determined after a simultaneous or separate coagulation activation via the intrinsic system using an APTT reagent, via the extrinsic system using tissue factor or by Factor Xa (Bertina R, loc cit ; Preda L et al,Thromb Res 60, 19-32 (1990); D'Angelo S et al, Thromb Res 77, 375-378 (1995)). Chromogenic activity assays for protein S have also been published, utilizing Factor IXa as activator and monitoring Factor Xa generation (van de Waart P, Woodhams BJ, *EP 567 636*) or thrombin generation (Rosén S, *EP 486 515*). In all these methods calcium ions are added as mentioned above.

The Factor V mutation (FV:Q^{506}) mutation in the Factor V molecule may be detected with molecular biology methods based upon the use of the polymerase chain reaction (PCR) technique (Bertina RM et al, *Nature* 369, 64-67 (1994)) or by methods in which the functional activity of APC is determined. Such activity methods may be coagulation-based (Dahlbäck B, *EP 608 235* ; Rosén et al, *Thromb Haemost* 72, 255-260 (1994)) and include the use of predilution of sample plasma with a plasma with no or a low Factor V activity (Dahlbäck B, *EP-A-94 905 908.3* ; Jorquera JI et al, *Lancet* 344, 1162 -1163 (1994); Svensson PJ et al, *Thromb Haemost* 77, 332-335 (1997)). The latter assay principle, vz a coagulation-based assay using predilution of sample plasma is also utilized in a commercial product, Coatest® APC Resistance V (Chromogenix AB). Alternatively, chromogenic methods may be used (Dahlbäck B, *EP 608 235* ; Rosén et al, *Thromb Haemost* 73, 1364, Abstract 1778 (1995), Nicolaes GAF et al, *Thromb Haemost* 76, 404-410 (1996)).

Since genetic defects in the Protein C anticoagulant pathway are found in about 25% of unselected patients with venous thromboembolism (VTE) and in about 50% of patients with thrombophilia, i.e. patients from families with an increased tendency to VTE, there is a need for a single test which detects all such abnormalities with a high sensitivity and specificity, i.e. a global (overall) test. One concept for a global test is based upon the ac-

tivation of Protein C in plasma with Protac®C and activation of coagulation via the intrinsic or extrinsic pathway (Bartl et al, *USP 5,001,069* ; Kraus M, *EP-A- 696 642*). Results obtained with a commercial kit application of this test, ProC Global (Behring Diagnostica, Marburg, Germany), in which intrinsic activation of coagulation is accomplished through addition of an APTT reagent, show a sensitivity for Protein C deficiency, Protein S deficiency and for FV:Q506 of, respectively, about 90%, 50-80% and more than 90% on analysis of healthy individuals and thrombotic patients (Dati F et al, *Clin Chem* 43, 1719-1723 (1997); Ruzicka K et al,*Thromb Res* 87, 501-510 (1997)). The specificity is about 50% in thrombotic cohorts (Dati F et al, loc cit), i.e. a substantial proportion of positive results are obtained, which can not be linked to known defects in components in the Protein C anticoagulant pathway, such as in protein C, protein S and Factor V. Thus, this test lacks sufficient specificity.

Furthermore, results from analysis of pregnant women lacking any of the known defects in the Protein C anticoagulant pathway are clearly different from analysis of normal healthy individuals (Rangård B, Wagner C, *Annals Hematol* 74: Supplement II, Abstract 74, A77 (1997); Siegemund A et al, *Annals Hematol* 74: Supplement II, Abstract 188, A105 (1997)), which necessitates separate ranges for these cohorts. This as yet uncharacterized interference limits the general applicability of the test. An alternative global method for the detection of defects in the protein C anticoagulant pathway, based upon activation of endogenous plasma Protein C by Protac C utilizes tissue factor as trigger of the coagulation (Preda L et al, *Blood Coag Fibrinol* 7, 465-469 (1996)). Also here, the sensitivity of the method is limited, especially for Protein S. Furthermore, different sample categories, e.g. pregnant and non-pregnant women, may require different approaches for evaluation of the results due to interferences not only related to components in the Protein C anticoagulant pathway.

For a global test to be useful as a screening test for known inherited defects in the Protein C anticoagulant pathway, i.e. Protein C deficiency, protein S deficiency and the FV:Q506 mutation, the sensitivity should be high, at least 90%, for all these defects. Furthermore the specificity should

be high, above 60%, suitably above 70% and preferably above 80% in order to considerably reduce the number of false positive results. The state-of-the-art methods do not provide a satisfactory solution to these requirements. For the development of improved methods for the specific determination of Protein C and Protein S activity and for determination of mutations in Factor V which affects its anticoagulant activity, it is also desirable to improve the resolution and specificity of these methods.

There is also a need to improve the stability of different components used as separate reagents or as reagents in kits comprising such methods.

Thus, the technical problem underlying the present invention is the provision of in vitro methods with improved sensitivity and specificity for screening and for the specific detection of defects in the Protein C anticoagulant pathway of blood coagulation in a human. A further recognized problem is to improve the stability of components in such methods.

Brief Description of the Invention

It has now been unexpectedly found that the above technical problems can be solved based upon the surprising finding that the addition of low levels of ions of divalent metals, such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Sr^{2+}, Cu^{2+} ions or of the monovalent Cu^{+} ion, in the presence of calcium ions enhances the anticoagulant activity of the Protein C anticoagulant pathway and provides for a high resolution between different levels of Protein C activity and Protein S activity, respectively, and a high discrimination for the presence of the FV:Q506 mutation, resulting in an improved sensitivity and specificity for detection of defects in components of the Protein C anticoagulant pathway with photometric and/or clotting methods. Thus, the invention also constitutes a new excellent global method for the Protein C anticoagulant pathway. In addition to that, divalent metal ions provide for an unexpected improvement of the stability of the reagents used either when used separately or when used in test kits for determining the anticoagulant activity of components of the Protein C anticoagulant pathway in

blood samples with photometric and/or clotting methods.

Thus, the above problems are solved by the characterizing features of the attached claims.

Subject-matter of the present invention thus is an in vitro photometric method for qualitative screening and quantitative determination of the functional activity of components of the Protein C anticoagulant pathway of blood coagulation, comprising measuring the conversion rate of an exogenous substrate by an enzyme, the activity of which is related to the Protein C anticoagulant activity, in a blood sample of a human comprising coagulation factors and said exogenous substrate after at least partial activation of coagulation through the intrinsic, extrinsic or common pathway and triggering coagulation by adding calcium ions; and comparing said conversion rate with the conversion rate of a normal human blood sample determined in the same way, said method being characterized by adding further metal(s) ions to said sample.

The present invention is thus concerned with a novel in vitro method for screening for, in a human, defects in the Protein C anticoagulant pathway due to e.g. Protein C deficiency, Protein S deficiency and Factor V mutations such as the FV:Q506 mutation, or other Factor V defects related to APC resistance and/or APC cofactor activity. Such a method may be designed for the specific detection of either of Protein C deficiency, Protein S deficiency or of mutations in Factor V/Factor Va which affect the cleavage rate by APC. One preferred embodiment of the present invention comprises a global test for the Protein C anticoagulant pathway.

The term "metal(s) ions" stands for the fact that ions of one or more of said further metals may be present. Preferred ions of said further metals are divalent metal ions or the ions of monovalent copper, such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Sr^{2+}, and/or Cu^{+} ions.

The term "blood sample" is defined to cover a blood sample, such as

whole blood, or a blood derived sample such as a blood plasma sample or a blood serum sample.

The term "photometric assay" is defined to cover colorimetric, fluorimetric and luminometric assay methods.

The term "coagulation factors" stands for such factors comprising components in the intrinsic, extrinsic and common pathway (procoagulant events see Figure 1) and in the Protein C anticoagulant pathway (anticoagulant events see Figure 1) and being either solely endogenous, i.e. being inherent in the blood sample, or comprising also the addition of such exogenous factors. Furthermore, phospholipid(s) may be added in the method when utilizing any of the intrinsic, extrinsic or common pathway for activation of coagulation.

Said novel method will thus allow improved screening and diagnosing of defects in the Protein C anticoagulant pathway in investigation of patients with thromboembolic diseases such as deep venous thrombosis and/or pulmonary embolism. In case a patient belongs to a family with hereditary thrombophilia, said novel method is also suitable for investigation of family members of such a patient in order to determine the possible inheritance of defects within said pathway. Said novel method is also suitable for diagnosing of defects in the Protein C anticoagulant pathway in investigation of patients before surgery, patients with trauma or in pregnant women or in women receiving oral contraceptive pills or hormone replacement therapy such as oestrogen therapy. Furthermore, global methods may be designed to allow the detection of known defects and of hitherto unrecognized defects in the Protein C anticoagulant pathway. The invention may also allow the design of specific photometric and/or clotting methods for such as yet unrecognized defects in said pathway.

Any of the above methods encompasses monitoring of conversion of a photometric exogenous substrate for either Factor Xa or thrombin, containing as leaving group a chromophore, a fluorophore or a luminophore. Examples of such photometrically measurable leaving groups are p-nitro-

aniline (pNA, chromophore) for use in colorimetric methods, e.g. naphthylamine and coumarin derivatives such as methylcoumarine, aminoisophthalic acid and its derivatives (fluorophores) for use in fluorimetric methods and e.g. isoluminolamide (luminophore) for use in luminometric methods.

The invention is most unexpected in light of the present knowledge in the field which, in fact, teaches that several divalent ions *increase the procoagulant activity* of certain vitamin K-dependent coagulation factors in the presence of calcium ions (see procoagulant events in Figure 1), so that it could not be expected that the addition of such further metal(s) ions could improve tests relating to the *anticoagulant activity*, specifically in the Protein C anticoagulant pathway

Thus, it is known that Mg^{2+} stimulates the activity of Factor IXa (Byrne et al, *J Biol Chem* 1980; 255: 1430-1435; Sekiya F et al, *J Biol Chem* 1995; 270: 14325-14331; Sekiya F et al, *J Biol Chem* 1996; 271: 8541-8544; Morita T et al. *Thromb Haemost* 1997; 78, Supplement: 430, Abstract PS-1755) and also enhances the activation rate of Factor IX by FXIa and tissue factor (Sekiya F et al, *loc sit 1995*, *loc cit* 1996; Morita T et al., *loc cit*). It was also shown that neither Protein C nor prothrombin, Factor VII and Factor X are responsive to Mg^{2+} (Sekiya F et al, *loc cit 1995*). Mg^{2+} has been shown to also stimulate the prothrombin activation by Factor Xa, phospholipid and calcium ions (Prendergast FG and Mann KG, *J Biol Chem* 1977; 252: 840-850), an effect which, however, may not be pronounced at calcium ion concentrations above 1 mmol/L (Sekiya F et al, *loc cit 1995*). Furthermore, it has been shown that Factor IX has a unique binding site for Mn^{2+} (Amphlett GW et al, *J Biol Chem* 1978; 253: 6774-6779), which site has been suggested to be identical with the Mg^{2+} binding site (Sekiya F et al, *loc cit 1995*).

Mn^{2+} ions have also been shown to enhance the binding of Factor IX to procoagulant phospholipids in the presence of calcium or Sr^{2+} ions, the latter thus also having a procoagulant effect (Liebman et al, *J Biol Chem*

1987; 262, 7605-7612).

Furthermore, Mg^{2+} and Mn^{2+} ions have been shown to increase the amidolytic activity of Factor VIIa, i.e. the cleavage rate of low molecular weight synthetic peptide substrates (Butenas S et al, *Biochemistry* 1994; 33: 3449-3456; Persson E, Petersen LC, *Eur J Biochem* 1995; 234: 293-300) whereas Zn^{2+} ions have been reported to have an inhibitory effect on the amidolytic activity of Factor VIIa but no effect on the amidolytic activity of Factor Xa, thrombin or activated Protein C (Pedersen AH et al,*Thromb Haemost* 1991; 65: 528-534). Mn^{2+} ions have also been shown to substitute for calcium ions in the activation of Factor X by Russell Viper Venom enzyme, albeit providing a lower activation rate (Bajaj P et al, *J Biol Chem* 1977; 252: 4758-4761).

The knowledge in the field also teaches that divalent metal ions such as Zn^{2+} and Cu^{2+} stimulate the autoactivation of Factor XII, a non-vitamin K-dependent coagulation factor (Shore et al, *Biochemistry* 1987; 26: 2250-2258; Bernardo MM et al, *J Biol Chem* 1993; 268: 12468-12476).

A further illustration that the present invention was unexpected is the knowledge which teaches that Mg^{2+} and Mn^{2+} stimulate the inhibition of APC by the two plasma protease inhibitors α_2-macroglobulin and plasmin inhibitor (Heeb MJ et al, *J Biol Chem* 1991; 266: 17606-17612). Thus, the addition of Mg^{2+} and Mn^{2+} under the conditions used results in a decreased anticoagulant activity of APC.

Therefore, the present invention providing an increased anticoagulant activity of the Protein C anticoagulant pathway through the use of metal(s) ions such as e.g. divalent metal ions or Cu^{+} in addition to calcium ions, could not be derived or expected from the state-of-the-art knowledge in the field.

Brief Description of the Drawings and Detailed Description of the Invention

In the following, the invention is disclosed more in detail making reference to the drawings enclosed, wherein:

Figure 1 shows a schematic representation of the blood coagulation system and its regulation;

Figure 2 shows a graphic representation of the results obtained in Example 2, i.e. the effects of the metal ions in a chromogenic Protein C assay;

Figure 3 shows a graphic representation of the effects obtained according to Example 5, namely the effect of Mg^{2+} and Mn^{2+} in a chromogenic Protein S assay;

Figure 4 assembles in a graphic representation the results obtained according to the method disclosed in Example 8, i.e. the effect of metal ions on discrimination for Protein S deficiency and for FV:Q506 in a global Protein C pathway assay using Factor Xa as activator;

Figure 5 shows a graphic representation of the effects obtained according to Example 12, namely the effect of Mg^{2+} in a global chromogenic assay for the detection of Protein C deficiency, Protein S deficiency and for FV:Q506 mutation using a recombinant tissue factor;

Figure 6 represents a graphic representation of the effects obtained according to Example 13, namely the effect of Mn^{2+} on the determination of free Protein S activity in a chromogenic thrombin generation assay;

Figure 7 shows a graphic representation of the effects obtained according to Example 14, namely the effect of Mg^{2+} and Mn^{2+} on the determination of Protein C activity in a chromogenic thrombin generation assay; and

Figure 8 provides a graphic representation of the effects obtained according to Example 15, namely the effect of Mg^{2+} and Mn^{2+} on the detection of Protein C deficiency, Protein S deficiency and for FV:Q506 mu-

tation in a global chromogenic method for using a recombinant tissue factor as activator of coagulation and monitoring thrombin generation.

A preferred embodiment of the present invention thus covers a method for the global screening for defects in the Protein C anticoagulant pathway of blood coagulation in a human, comprising

A) incubating a blood sample of said human comprising coagulation factors with:

1) an activator for the Protein C in said sample,

2) a suitable coagulation activator,

3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

4) calcium ions, and

5) said further metal(s) ions;

B) determining the conversion rate of said exogenous substrate; and

C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

A further preferred embodiment of the present invention relates to a method for the determination of free Protein S activity, comprising:

A) incubating said blood sample comprising coagulation factors with:

1) exogenous activated Protein C or exogenous Protein C together with an activator of Protein C,

2) a suitable coagulation activator,

3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

4) calcium ions, and

5) said further metal(s) ions;

B) determining the conversion rate of said exogenous substrate; and

C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

Another preferred embodiment of the present invention relates to a method for the determination of Protein C activity, comprising:

A) incubating a blood sample of said human comprising coagulation factors with:

1) an activator for the Protein C in said sample,

2) a suitable coagulation activator,

3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group, and

4) calcium ions, and

5) said further metal(s) ions;

B) determining the conversion rate of said exogenous substrate; and

C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

A fourth further preferred embodiment of the present invention covers a method for screening for Factor V mutation(s) in a blood sample of said human, comprising:

A) incubating a blood sample of said human comprising coagulation factors with:

1) exogenous activated Protein C, or exogenous Protein C together with an activator of Protein C, or an activator for endogenous Protein C,

2) a suitable coagulation activator,

3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

4) calcium ions, and

5) said further metal(s) ions;

B) determining the conversion rate of said exogenous substrate; and

C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

In the above preferred methods for global screening for defects in the Protein C anticoagulant pathway, for the determination of free Protein S activity or Protein C activity or for screening for Factor V mutation(s) such as

the FV:Q506 mutation in a blood sample, step A) comprises incubating the blood sample of said human comprising coagulation factors in the presence of the added further metal(s) ions used according to the present invention with

1) an activator for the Protein C in said sample to provide activation of endogenous Protein C, or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C,

2) a suitable coagulation activator to provide at least partial activation of coagulation,

3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group to provide for monitoring Factor X_a or thrombin activity and

4) calcium ions to trigger coagulation.

These steps 1) to 4) can be performed separately and/or simultaneously, i.e. in different sequential combinations providing for so-called "one-stage" to "four-stage" methods as follows:

In a one-stage method all components necessary for performing steps 1) to 4) above are added simultaneously.

In two-stage methods a) the components for steps 1) and 2) may first be combined followed by simultaneous addition of the exogenous substrate with calcium ions (3) and 4)), or b) all the components but for calcium ions (1)-3)) may be added simultaneously, the addition of calcium ions (4) then comprising the second step,or c) the components for step 1), 2) and 4) may be included simultaneously and step 3) be performed as a separate step.

In three-stage methods a) steps 1) and 2) are combined and steps 3) and 4) performed as separate steps, or b) steps 1) and 2) are performed as separate steps and steps 3) and 4) are performed simultaneously, or c) steps 2) and 3) are performed as separate steps, and steps 1) and 4) are performed simultaneously, or d) step 1) is performed as a separate step, steps 2) and 4) are performed simultaneously, followed by step 3).

In four-stage methods steps 1) to 4) are performed as separate steps in the order as described or in any other different order.

All one-, two-, three- or four-stage methods may be used in chromogenic, fluorimetric and luminometric methods.

Other embodiments of the invention comprise clotting methods, utilizing activation through the intrinsic, extrinsic or common pathway.

For any method, said further metal(s) ions used according to the present invention may be added either initially or at a later stage to any of said reagents. In applications where endogenous Protein C is activated by a Protein C activator, such as in methods for Protein C activity and global methods for the Protein C anticoagulant pathway, one preferred mode of the invention is to include said further metal(s) ions in the Protein C activation step, for example when Protac®C is used as the Protein C activator.

Specifically, the invention concerns the addition of divalent metal ions or of the monovalent Cu^+ ion in view of increasing the resolution between different levels of, respectively, Protein C activity and Protein S activity as well as providing a high discrimination for the presence of the FV:Q^{506} mutation, resulting in an improved sensitivity and specificity for detection of defects in components of the Protein C anticoagulant pathway of blood coagulation. The range of concentrations of metal ions within which the anticoagulant activity of the Protein C system is stimulated varies with respect to the actual metal ion. The optimal concentration for Mg^{2+} ions is considerably higher than that for other metal ions. The concentration range for metal ions other than Mg^{2+} comprises 1 µmol/L - 2mmol/L, suitably 5 - 400 µmol/L and preferably 10 - 80 µmol/L. For Mg^{2+} ions, the concentration range comprises 20 µmol/L - 10 mmol/L, suitably 100 µmol/L - 2 mmol/L and preferably 200 µmol/L - 1 mmol/L.

The counter ion should be selected in such a way to allow the above

described available concentrations of metal ions. Suitable counter ions are mono-, di- and trivalent anions, preferably mono- and divalent anions, such as chloride, sulphate and nitrate anions. Metal ions could also be provided in form of a metal ion complex with protein(s) such as blood protein or on a solid surface such as a metal ion coated wall of a reaction vessel.

For any application of the invention, photometric methods are used for monitoring the anticoagulant activity of components in the Protein C anticoagulant pathway.

In photometric methods, synthetic substrates with colorimetric, fluorimetric or luminometric leaving groups are used, which substrates preferably should be selective for Factor Xa or for thrombin. Since the generation of Factor Xa and thrombin according to the invention is influenced by the Protein C anticoagulant activity in the reaction mixture, containing a test sample from an individual, the measurement of the conversion rate of said substrates by said enzymes is suitably performed for determination of said Protein C anticoagulant activity. The measurement of said conversion rate is suitably compared with the corresponding conversion rate obtained when using a normal human plasma pool as a test sample. The conversion rate may be measured kinetically, i.e. by monitoring the change in optical density (OD) versus time, expressed as e.g. ΔOD/min, or measured after a fixed incubation time, expressed as OD.

The determination of the conversion rate of synthetic substrates is performed with instruments suitable for monitoring the release of the leaving group from the actual substrate chosen. When the conversion rate is determined in a microplate reader, it is suitable to perform readings in the so called dual wavelength mode in order to eliminate possible differences between microplate wells. In such readings, one wavelength is selected for detecting the release of the leaving group whereas the other wavelength is selected in a wavelength range where the released leaving group does not have any appreciable absorbance. When a colorimetric leaving group such as paranitroaniline (pNA) is chosen, a suitable dual wavelength reading is per-

formed at 405 and 490 nanometers, expressed as $OD_{405-490nm}$.

A further aspect of the invention is the use of diluted blood samples in said photometric methods in order to avoid interference of blood sample components in the test sample. The final concentration, i.e. the concentration in the sample the optical density of which is determined, of sample may vary depending on the actual method used. For colorimetric methods said blood sample concentration may be below 10% and preferably below 5%.

Any activator of endogenous Protein C may be used such as thrombin with or without thrombomodulin, both from human or non-human sources or being produced by recombinant technology as wild-type proteins or as modified polypeptides to provide the suitable functional property, or alternatively a snake venom enzyme which activates Protein C. Suitable snake venom enzymes are preferably selected from the Agkistrodon snake family and may be added as crude venom or in a purified state as the product Protac®C. Suitable snake venom enzymes may also be produced by recombinant technology as wild-type proteins or as modified polypeptides to provide the suitable functional property. The concentration of the Protein C activator may vary depending on the actual conditions used. Thus, for the activator Protac®C the concentration may vary between 1×10^{-3} and 1 U/mL, preferably 2×10^{-3} and 0.3 U/mL during the activation of Protein C in a global method for the Protein C anticoagulant pathway or in specific methods for Protein C and free Protein S activity or for a method for detection of mutations in Factor V/Factor Va which affect the cleavage rate by APC.

According to a further preferred embodiment of the present invention, the activation of Protein C in the blood sample precedes or occurs simultaneously with activation of coagulation.

In the methods of the present invention, a suitable activator of coagulation is being used. Such activators may be selected to activate the so-called intrinsic, extrinsic or common pathway.

Activation through the intrinsic system may be accomplished with an APTT reagent containing a suitable contact activator, or with the separate addition of a contact activator. As such activators of the intrinsic pathway, suitable compositions of phospholipids and contact activators may be used. As contact activators, ellagic acid, collagen or collagen related substances or various forms of silica (kaolin, micronized silica, colloidal silica) may be used. Alternatively Factor XIIa, Factor XIa or Factor IXa may be used in combination with phospholipids as activators of the intrinsic pathway rather than using a contact activator. Optionally, components such as prothrombin, Factor VIII/Factor VIIIa and Factor X may be added. Photometric substrates selective for Factor Xa or thrombin are used.

Activation through the extrinsic system may be accomplished by tissue factor from human or non-human tissues or produced by recombinant technology as a wild-type protein or as a suitably modified polypeptide with or without the addition of Factor VII/Factor VIIa. Alternatively, activation may be accomplished by Factor VIIa in combination with said phospholipids. Optionally, reagents such as prothrombin, Factor V/Va, Factor IX and Factor X may be added. Photometric substrates selective for FXa or thrombin are used.

Activation of the common pathway may be accomplished by addition of exogenous Factor Xa or by exogenous Factor X in combination with an exogenous activator of Factor X, such as a snake venom enzyme, e.g. a snake venom enzyme from Russell Viperii. Alternatively, said exogenous activator of Factor X may be added for activation of endogenous Factor X. Optionally, prothrombin and/or Factor V/Va may be added. Photometric substrates selective for thrombin are used.

In theabove described modes of the at least partial activation of coagulation according to the intrinsic, extrinsic or common pathway phospholipids may be added as a mixture of synthetic phospholipids and/or purified phospholipids or as crude extracts from biological sources such as e.g. brain,platelet, placenta, egg yolk or soybean.

Generally, interferences due to e.g. variable functional levels of components in the sample may be minimized by including a suitable amount of plasma deficient of the Protein C anticoagulant pathway component to be measured. Such plasmas may be deficient of e.g. Protein C, Protein S or Factor V. In case of a global method for the Protein C anticoagulant pathway, a plasma deficient of Protein C, Protein S and Factor V may be added.

In case of a method for Protein S activity or a method for detection of a Factor V mutation which affects the degradation of Factor V/Va by APC, exogenous Protein C may be added as a plasma deficient of Protein S and Factor V, respectively.

Protein S may be added in methods for Protein C activity or for detection of said mutation(s) in Factor V.

In case of protein C and protein S methods, Factor V/Factor Va may also be added to minimize interference from mutated Factor V present in the sample, vz mutations which affect the degradation of Factor V/Va by APC.

The above-mentioned coagulation factors and components of the Protein C anticoagulant pathway, vz Factor XIIa, Factor XIa, Factor IX/IXa, Factor VIII/VIIIa, Factor VII/VIIa, Factor X/Xa, Factor V/Va, prothrombin, Protein C/APC and Protein S are of human or non-human origin, suitably bovine or human origin. Said coagulation factors may also be produced by recombinant technology as wild-type or as modified polypeptides with suitable biological activity.

In order to prevent fibrin gel formation, a fibrin polymerization inhibitor may be added to the reaction mixture such as Gly-Pro-Arg-Pro.

In chromogenic methods, any chromogenic substrates for Factor Xa may be used, such as e.g. Benzoyl-Ile-Glu-Gly-Arg-pNA (S-2222, Chromogenix AB, Mölndal, Sweden), N-α-Z-D-Arg-Gly-Arg-pNA (S-2765, Chromo-

genix AB), CH_3SO_2-D-Leu-Gly-Arg-pNA (CBS 31.39, Stago Diagnostica) and MeO-CO-D-CHG-Gly-Arg-pNA (Spectrozyme Xa, American Diagnostica, Greenwich, USA). Correspondingly, any chromogenic substrates for thrombin may be used, e.g. H-D-Phe-Pip-Arg-pNA (S-2238, Chromogenix AB), pyroGlu-Pro-Arg-PNA (S-2366, Chromogenix AB), D-Ala-Pro-Arg-pNA (S-2846, Chromogenix AB), Z-D-Arg-Sarc-Arg-pNA (S-2796, Chromogenix AB), AcOH*H-D-CHG-But-Arg-pNA (CBS 34.47, Stago) and H-D-HHT-Ala-Arg-pNA (Spectrozyme TH, American Diagnostica).

A further subject matter of the present invention is a kit for use in the the above methods comprising the following components:

a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;
b) a suitable coagulation activator;
c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;
d) calcium ions; and
e) said further metal(s) ions;

in separate containers and/or in containers comprising mixtures of at least two of said components in aqueous solution or in lyophilized form. .

A further preferred embodiment of the present invention relates to a kit for use in the the above methods comprising the following components:

a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;
b) a suitable coagulation activator;
c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;
d) calcium ions;
e) said further metal(s) ions;
f) coagulation factors; and

in separate containers and/or in containers comprising mixtures of at least two of said components in aqueous solution or in lyophilized form.

A still further embodiment of the present invention comprises a reagent for use in the above methods comprising said further metal(s) ions and at least one of the following components a) to e):

a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;

b) a suitable coagulation activator;

c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;

d) calcium ions; and

e) coagulation factors;

in one container in aqueous solution or in lyophilized form.

According to a preferred embodiment, said reagent comprises at least two of said components a) to e) and said further metal(s) ions in one container in aqueous solution or in lyophilized form.

One preferred embodiment of said reagent comprises activated Protein C, with or without calcium ions and said further metal(s) ions. One further preferred embodiment of said reagent comprises coagulation factors andProtein C activators and said further metal(s) ions, if desirable in combination with phospholipid(s). One further embodiment comprises Factor IX/IXa, Factor X/Xa and/or calcium ions and said further metal(s) ions or said further metal(s) ions in combination with Factor V/Va, Protein C and prothrombin. Optionally Factor VIII/VIIIa and/or thrombin may be combined with said further metal(s) ions. In a further embodiment said further metal(s) ions are combined with a Protein C activator, such as Protac®C or thrombin/thrombomodulin. Said reagent embodiments are suitably comprised in a container in aqueous solution or in lyophilized form.

A wide range of concentrations of reactants can be used in the methods of the present invention. The following, Table 1 presents suitable and preferred ranges for the various components used in the method and contained in the kits and reagents according to the present invention.

Table 1

Parameter	Final concentration in final reaction medium
Blood sample	0.02 - 10 %, preferably 0.1-5 % (v/v)
FIX/FIXa	1×10^{-15} - 1×10^{-6} mol/L
FX/FXa	1×10^{-15} - 5×10^{-7} mol/L
FV/FVa	1×10^{-12} - 10^{-7} mol/L, preferably 2×10^{-10} - 5×10^{-8} mol/L
FVII/FVIIa	1×10^{-15} - 2×10^{-8} mol/L
FVIII/FVIIIa	1×10^{-4} - 5×10^{-1} IU/mL
Prothrombin	1×10^{-9} - 5×10^{-7} mol/L
Thrombin	1×10^{-15} - 1×10^{-8} mol/L
Ca^{2+} ions	0.5 - 20 mmol/L, preferably 1 - 10 mmol/L
Mg^{2+} ions	20 µmol/L - 10 mmol/L, suitably 100 µmol/L - 2 mmol/L and preferably 200 µmol/L - 1 mmol/L.
Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Sr^{2+} and/or Cu^{+} ions	1 µmol/L - 2mmol/L, suitably 5 - 400 µmol/L and preferably 10 - 80 µmol/L
Protein C/APC	1×10^{-10} - 1×10^{-7} mol/L, preferably 5×10^{-10} - 1×10^{-8} mol/L
Protac®C	1×10^{-3} - 1 U/mL, preferably 2×10^{-3} - 0.3 U/mL
Protein S	10^{-9} - 5×10^{-7} mol/L
Tissue factor	10^{-8} - 10^{-5} g/L, preferably 5×10^{-8} - 10^{-6} g/L
Thrombin/ thrombomodulin	10^{-11} - 10^{-8} mol/L
Phospholipid	1×10^{-6} - 3×10^{-4} mol/L
Fibrin polymerization inhibitor	Range dependent of substance used
Chromogenic substrate	10^{-5} - 5×10^{-3} mol/L
pH	6.5 - 9.5, preferably 7-8.5
Ionic strength	0-0.6, preferably 0.01-0.25

Suitable embodiments include preparation in aqueous solution or lyophilization in a container of one or more components presented in the above Table 1 such as a protein with and without metal(s) ions, optionally in the presence of phospholipid to provide suitable reagents for use in the method according to the invention. Said embodiments may comprise e.g. Protein C or APC and said further metal(s) ions and with calcium ions and with or without an active enzyme such as Factor IXa or Factor Xa. Other embodiments may comprise metal(s) ions with any or a combination of Factor V/Va, Protein S, prothrombin, Factor X, Factor VIII/VIIIa, thrombin. A further embodiment may comprise a Protein C activator such as Protac®C or thrombin/thrombomodulin with metal(s) ions.

The methods of the present invention allow for convenient reaction times such as 1-10 min, preferably 2-5 min, to provide for easy applicability to automated coagulation instruments.

The invention is also concerned with kits and reagents for use in the above in vitro methods for screening and diagnosing for Protein C and Protein S deficiency and for mutations in Factor V which affect the anticoagulant activity of APC. The invention is further concerned with a kit for an in vitro method for screening and diagnosing for defects in the Protein C pathway, caused by e.g. Protein C deficiency, Protein S deficiency and Factor V mutations. Said kits comprise a suitable selection of components listed in Table 1, prefereably present as reagents in one or more container(s) comprising said components in aqueous solution or in lyophilized form.

The invention is illustrated by the following examples which, however, in no way restrict the scope of the claims.

Example 1

Effect of manganese and magnesium ions on the determination of Protein C activity in a three-stage chromogenic thrombin generation assay using the Protein C activator Protac®C.

Samples: Protein C deficient plasma (Biopool, Umeå, Sweden) with and without addition of purified human Protein C (Chromogenix AB) to yield 0, 0.1, 0.5 and 1.0 IU/mL of Protein C.

Sample dilution: 1:41 in 25 mmol/L Tris-HCl pH 7.6, 20 mmol/L NaCl, 0.2% bovine serum albumin.

Protein C activator: Protac®C was used as a stock solution containing 10 U/mL. Final concentration during activation of Protein C = 0.17 U/mL. Mg^{2+} and Mn^{2+} ions were added to yield final concentrations during activation of Protein C of 0.4 and 0.04 mmol/L, respectively.

Reagent 1: Bovine Factor IXa (Enzyme Research, South Bend, Ill., USA), 180 pmol/L
Bovine FX (Chromogenix AB), 0.3 U/mL

Reagent 2: Phospholipids* (Chromogenix AB), 60 µmol/L
Gly-Pro-Arg-Pro, 0.36 mg/mL (polymerization inhibitor)
Human Factor V, 0.2 U/mL
$CaCl_2$, 6 and 24 mmol/L (final conc. in assay = 1.5 and 6 mmol/L)

*) A mixture of purified phospholipids containing 43% phosphatidylcholine, 27% phosphatidylserine and 30% sphingomyelin.

Chromogenic thrombin substrate: S-2796 (Chromogenix AB), 2 mmol/L

Assay in a microplate:

This assay is carried out as a three-stage method comprising, in the first stage, combining 50 µL of the diluted plasma with 50 µl of the Protein C activator Protac®C and incubating this mixture for three minutes at 37°C, whereafter coagulation is activated by adding 50 µL of Reagent 1 and 50 µL of Reagent 2 and incubating the mixture for five minutes at 37°C, whereafter, in the third stage, the substrate hydrolysis is carried out by adding 50 µL of the chromogenic thrombin substrate S-2796 and incubating for four minutes at 37°C. The reaction is then terminated by lowering the pH

through addition of 50µL of 20% acetic acid. Thereafter the optical density (OD) of the samples in the microwells is recorded at 405 and 490 nm and the difference in optical density between 405 and 490 nm, $OD_{405-490nm}$, is calculated. This three-stage reaction is schematically shown as follows:

Protein C activation:	Plasma dilution	50 µL
	Protein C activator	50 µL
	3 min, 37°C	
Coagulation activation	Reagent 1	50 µL
	Reagent 2	50 µL
	5 min, 37°C	
Substrate hydrolysis	S-2796	50 µL
	4 min, 37°C	
	HOAc, 20%	50 µL

Recording of $OD_{405-490nm}$

Results:

	Protein C, IU/mL			
	0	0.1	0.5	1.0
Ca^{2+}, 6 mmol/L	0.542	0.515	0.503	0.467
Ca^{2+}, 1.5 mmol/L + Mn^{2+}, 0.04 mmol/L	0.564	0.441	0.231	0.076
Ca^{2+}, 1.5 mmol/L + Mg^{2+}, 0.4 mmol/L	0.541	0.441	0.230	0.069

The results demonstrate that by including manganese and magnesium ions in a reaction system containing calcium ions, a strong enhancement of the anticoagulant activity is obtained, manifested by the fact that increasing concentrations of Protein C in the samples result in a much de-

creased absorbance, i.e. a much decreased thrombin generation. In contrast, in the presence of calcium ions alone, there is a much lower resolution in absorbance, i.e. in thrombin generation, at increasing Protein C concentrations. Thus, the addition of further metal ions constitutes an improved method for determination of Protein C activity.

Example 2

Effect of different metal ions on determination of Protein C activity in a three-stage thrombin generation assay using a four-fold lower concentration of Protein C activator.

Experimental conditions as in Example 1, except for the use of a final concentration of the Protein C activator (Protac®C) of 0.043 U/mL. Mg^{2+},Mn^{2+}, Zn^{2+} and Ni^{2+} ions were added to yield final concentrations during activation of Protein C of 0.4 mmol/L (Mg^{2+}) or 0.04 mmol/L. Zn^{2+} ions, Mn^{2+} ions and Cu^{2+} ions were also added to yield a final concentration of 0.08 mmol/L. Ca^{2+} was also used at final concentrations of 1.5 mmol/L and 6.6 mmol/L in the absence of other metal ions.

Results: The results are shown in the table below with all primary data, which also includes a comparison between final concentrations of 0.04 and 0.08 mmol/L for Mn^{2+} and Zn^{2+}.

	Protein C, IU/mL			
	0	0.1	0.5	1.0
Ca^{2+}, 6 mmol/L	0.652	0.633	0.559	0.505
Ca^{2+}, 1.5 mmol/L	0.640	0.585	0.504	0.438
Ca^{2+}, 1.5 mmol/L + Mn^{2+}, 0.04 mmol/L	0.725	0.689	0.503	0.237
Ca^{2+}, 1.5 mmol/L + Mn^{2+}, 0.08 mmol/L	0.627	0.469	0.145	0.056
Ca^{2+}, 1.5 mmol/L +				

	Mg^{2+}, 0.4 mmol/L	0.627	0.583	0.361	0.123
	Ca^{2+}, 1.5 mmol/L + Zn^{2+}, 0.04 mmol/L	0.513	0.446	0.334	0.129
	Ca^{2+}, 1.5 mmol/L + Zn^{2+}, 0.08 mmol/L	0.421	-	0.189	0.051
	Ca^{2+}, 1.5 mmol/L + Ni^{2+}, 0.04 mmol/L	0.594	0.437	0.173	0.047
	Ca^{2+}, 1.5 mmol/L + Cu^{2+}, 0.08 mmol/L	0.487	0.425	0.348	0.099

The above results further graphically shown in Figure 2 demonstrate that many different metal ions provide an enhancement of the anticoagulant activity and also that a calcium concentration of 1.5 mmol/L in the absence of any other added further metal ions lacks the anticoagulation enhancement property. Furthermore, the concentration of Protac®C is not critical since the use of a four-fold lower concentration of this component still results in a pronounced anticoagulant activity in the presence of added metal ions.

Example 3

Effect of metal ions on determination of Protein C activity in a two-stage thrombin generation assay.

These experimental details are as described in Example 1, with the following exceptions:

- the final concentration of the Protein C activator (Protac®C) was 0.043 U/mL during activation of Protein C.
- the chromogenic thrombin substrate used was S-2846 (Chromogenix AB),
- the chromogenic substrate was included in Reagent 1,
- purified human Protein C was added to Protein C deficiency plasma to yield 0, 0.1 and 0.5 IU/mL,

metal ions tested: Mn^{2+} and Mg^{2+}.

Results, expressed as $OD_{405-490nm}$:

a) Mn^{2+} ions

	Protein C, IU/mL		
	0	0.1	0.5
Ca^{2+}, 6 mmol/L	1.18	1.07	0.669
Ca^{2+}, 1.5 mmol/L + Mn^{2+}, 0.04 mmol/L	1.36	1.17	0.258

b) Mg^{2+} ions

	Protein C, IU/mL		
	0	0.1	0.5
Ca^{2+}, 6 mmol/L	1.24	1.05	0.554
Ca^{2+}, 1.5 mmol/L + Mg^{2+}, 0.4 mmol/L	1.45	1.15	0.215

These results show that a significantly higher resolution for the different Protein C activities is obtained when Mn^{2+} and Mg^{2+} ions are added to a final concentration of 0.04 mmol/L and 0.4 mmol/L, respectively, thus constituting an improved two-stage method for determination of Protein C activity.

Example 4

Effect of manganese ions on determination of Protein C activity in a two-stage thrombin generation assay using a phospholipid emulsion from bovine brain.

Phospholipid source: Cephotest (Nycomed, Oslo, Norway)

Experimental details as in Example 3. The final concentration of Cephotest was 3% (v/v).

Results, expressed as $OD_{405-490nm}$:

	Protein C, IU/mL		
	0	0.1	0.5
Ca^{2+}, 6 mmol/L	1.09	0.813	0.375
Ca^{2+}, 1.5 mmol/L + Mn^{2+}, 0.04 mmol/L	1.366	0.996	0.163

The results show that the same enhancing effect on the Protein C anticoagulant activity is obtained with a crude phospholipid extract from an animal tissue source. Hence, the source of phospholipid is not critical.

Example 5

Effect of manganese and magnesium ions on determination of free Protein S activity in a chromogenic Factor Xa generation assay.

Sample: Protein S deficient plasma (Biopool) with or without addition of purified human Protein S to yield 0%, 25% and 100% Protein S.

Sample dilution: 1:61 in 50 mmol/L Tris buffer pH 8.2, 0.15 mol/L NaCl, 0.2% BSA

Factor reagent (concentration in assay before substrate addition):

Bovine FIXa (4 mU/mL)

Bovine FX (0.3 U/mL)

Human FVIII (0.02 U/mL)

Human FV (0.02 U/mL)

Human prothrombin (0.01 U/mL)

Phospholipids (21 µmol/L)

Mg^{2+} (0.4 mmol/L) or Mn^{2+} (0.04 mmol/L) or no addition

Medium: 10 mmol/L MES pH 6.0, 0.15 mol/L NaCl, 0.2% BSA

Start reagent

Human APC (0.35 µg/mL)

$CaCl_2$ (1.5 mmol/L)

Chromogenic Factor Xa substrate: S-2765 (Chromogenix AB), 1.8 mmol/L.

For carrying out the assay 50 µL of the diluted plasma sample was mixed with 50 µL of the above Factor reagent, whereafter the mixture was incubated for three minutes at 37°C. Thereafter, 50 µL of the Start reagent comprising human APC and calcium chloride was added and the mixture incubated for four minutes at 37°C. Following that, 50 µL of the chromogenic substrate S-2765 was added to and the reaction mixture incubated for two minutes at 37°C, whereafter 50 µL acetic acid was addedto terminate the reaction. The absorbance of the sample was then determined according to Example 1 and expressed as $OD_{405-490\ nm}$.

Assay:		
	Factor reagent	50 µL
	Sample dilution	50 µL
	Incubation 3 min, 37°C	
	$APC/CaCl_2$	50 µL
	4 min, 37°C	
	S-2765, 1.8 mmol/L	50 µL
	2 min, 37°C	
	HOAc, 20%	50 µL

Results: All primary data are listed in the table below and also illustrated in Figure 3.

	Free Protein S, %		
	0	25	100
Ca^{2+}, 1.5 mmol/L	0.857	0.642	0.364
Ca^{2+}, 1.5 mmol/L + Mn^{2+}, 0.04 mmol/L	1.53	1.34	0.745
Ca^{2+}, 1.5 mmol/L + Mg^{2+}, 0.4 mmol/L	1.053	0.851	0.378

Figure 3 shows that the addition of Mg^{2+} or Mn^{2+} ions results in a greater resolution, (i.e. a greater slope of the curve) as well as in a more linear dose response when compared to the use of Ca^{2+} alone, thus constitut-

ing an improved method for determination of Protein S activity.

Example 6

Effect of strontium ions on the determination of free Protein S activity in a chromogenic Factor Xa generation assay.

The experimental details are as disclosed in Example 5 but with the Factor reagent stored for one hour before assay.

Results: Absorbances expressed as $OD_{405-490nm}$

	Free Protein S, %		
	0	25	100
Ca^{2+}, 1.5 mmol/L	0.673	0.488	0.258
Ca^{2+}, 1.5 mmol/L + Sr^{2+}, 0.4 mmol/L	0.848	0.611	0.276

The results show that a higher resolution for various Protein S activity levels is obtained on addition of Sr^{2+} ions, supporting the enhancing effect of Sr^{2+} on the Protein C anticoagulant pathway activity.

Example 7

Effect of metal ions on the detection of Protein S deficiency in a global chromogenic method for the Protein C anticoagulant pathway, using tissue factor as activator of coagulation and monitoring thrombin generation.

Sample: Normal human plasma pool and Protein S deficient plasma (Biopool, Umeå, Sweden)

Sample dilution: 1:21 in 25 mmol/L Tris-HCl pH 7.6, 20 mmol/L NaCl, 0.2% bovine serum albumin.

Protein C activator: Protein C activator (Protac®C) from Coamatic Protein C. was reconstituted in 7.2 mL according to the kit package insert and then

diluted in 25 mmol/L Tris-HCl pH 7.6, 20 mmol/L NaCl, 0.2% bovine serum albumin to yield a concentration during Protein C activation of 0.02 U/mL. Human prothrombin (Chromogenix AB) was added to yield a final concentration after addition of tissue factor of 1.5 µg/mL.

The analysis was performed with or without Mg^{2+} ions added to the Protac®C solution.

Reagent:

Tissue factor: Thromborel (Behringwerke, Marburg, Germany). Reconstituted in 2 mL water according to the manufacturer, thereafter diluted in 25 mmol/L Tris-HCl pH 7.6, 20 mmol/L NaCl, 0.2% bovine serum albumin to yield a final concentration during activation of coagulation of 0.033% (v/v).

Phospholipids (43% phosphatidylcholine, 27% phosphatidylserine and 30% sphingomyelin): Final concentration during activation of coagulation of 16.7 µmol/L.

$CaCl_2$: 6.6 mmol/L final concentration during activation of coagulation.

Chromogenic thrombin substrate: S-2796 (Chromogenix AB), 1.8 mmol/L

For carrying out the assay 50 µL of the diluted plasma sample was mixed with 50 µL of the Protein C activator, whereafter the mixture was incubated for two minutes at 37°C. Thereafter, 50 µL of the reagent comprising the tissue factor was added and the mixture incubated for two minutes at 37°C. Following that, 50 µL of the chromogenic substrate S-2796 was added and the reaction mixture incubated for four minutes at 37°C, whereafter 50 µL acetic acid solution was added to terminate the reaction. The absorbance of the sample was then determined according to Example 1 and expressed as $OD_{405-490\ nm}$.

Microplate

Assay:	Sample dilution	50 µL
	Protac C activator	50 µL
	2 min, 37°C	

Reagent		50 µL
2 min, 37°C		
S-2796		50 µL
4 min, 37°C		
HOAc, 20 %		50 µL

Results:

	Normal plasma	Protein S def. plasma
Ca^{2+}, 6.6 mmol/L	0.26	0.53
Ca^{2+}, 6.6 mmol/L + Mg^{2+}, 0.4 mmol/L	0.29	0.75

The results show that the addition of magnesium ions to calcium ions brings about a higher resolution at different Protein S activity levels thus improving detection of Protein S deficiency.

Example 8

Effect of metal ions on resolution between different levels of free Protein S and for detection of FV:Q506 in a global method for the Protein C anticoagulant pathway using Factor Xa as activator of coagulation and monitoring thrombin generation.

Experimental details as in Example 7, but with bovine Factor Xa (Chromogenix AB) used instead of tissue factor as activator of coagulation. Concentration of Factor Xa = 1.4 ng/mL during activation. Furthermore, a stock solution of Protac®C, containing 10 U/mL, was used, which was then diluted in 25 mmol/L Tris-HCl pH 8.4, 0.2% bovine serum albumin to yield a concentration during protein C activation of 0.02 U/mL.

Samples:	
100% protein S =	normal human plasma pool
0% protein S =	protein S deficient plasma
25% protein S =	protein S deficient plasma + 2.5 µg/mL purified human protein S.

Furthermore, a sample from an individual with heterozygosity for the factor V mutation (FV:R506Q) was analysed.

Results: See Figure 4. The table below presents all primary data expressed as $OD_{405-490nm}$.

Sample	Only Ca^{2+}	Ca^{2+} + 0.04 mM Mn^{2+}	Ca^{2+} + 0.4 mM Mg^{2+}
100% Protein S	0.222	0.309	0.414
25% Protein S	0.411	0.485	0.667
0% Protein S	0.650	0.975	1.147
FV:R506Q	0.402	0.693	0.761

The results show that a higher resolution is obtained for Protein S deficiency in the 0 - 100% range as well as a high discrimination for the FV:Q506 mutation when Mg^{2+} or Mn^{2+} ions are included in the reaction mixture, thus proving the beneficial use of added metal ions in a global chromogenic method.

Example 9

Comparison between a global chromogenic method according to the invention , using Factor Xa as coagulation activator, with a global clotting method according to theprior art using APTT as coagulation activator, regarding resolution between different levels of, respectively, Protein C and Protein S activity and regarding analysis of plasma from pregnant women.

Samples: Human normal plasma pool (NPL), three plasmas from healthy individuals (N1-N3), four plasmas from pregnant women (P1-P4) and plasmas with 0% and 50% deficiency of Protein C and Protein S, respectively, the 50% levels being prepared by adding purified human Protein C (Chromogenix AB) or Protein S (Chromogenix AB) to plasmas deficient in either Protein C or S (both from Biopool AB).

Global chromogenic assay: Experimental details and assay, see Examples 7 and 8. A stock solution of Protac®C, containing 10 U/mL, was used which

was then diluted to yield a final concentration during activation of Protein C = 0.02 U/mL. Metal ion used = Mn^{2+}, which was added to the Protac®C solution to yield 0.04 mmol/L in the Protein C activation step. The analysis was performed in a microplate and the $OD_{405-490nm}$ was determined as described in Example 1. A high $OD_{405-490}$ corresponds to pronounced thrombin formation and thus an impaired Protein C anticoagulant pathway activity.

Global clotting assay using APTT reagent: APTT reagent from Coatest® APC Resistance was used at a final phospholipid concentration of 33 µmol/L during coagulation activation. For activation of Protein C, the same Protac®C stock solution and dilution medium was used as for the chromogenic assay. Final concentration during activation of Protein C = 0.083 U/mL. The analysis was performed in a ST-4 coagulation analyzer (Diagnostica Stago).

Assay:	Plasma sample	50 µL
	Protac®C or buffer	50 µL
	APTT reagent	50 µL
	Activation for 3 min, 37°C	
	Ca^{2+}, 25 mmol/L	50 µL

The clotting time in seconds was determined in the presence (CT+) and absence (CT-) of Protac®C and a clot time ratio (CTR) was calculated as CTR = CT+/CT-. A low CTR corresponds to a pronounced thrombin formation also in the presence of Protac®C and hence an impaired Protein C anticoagulant pathway activity.

Results:

Sample	Chromogenic	APTT
	$OD_{405-490}$	CTR
NPL	0.202	3.77
N1	0.183	5.17

1	N2	0.182	3.50
	N3	0.186	4.85
	P1	0.221	3.15
	P2	0.298	2.21
5	P3	0.239	2.60
	P4	0.259	2.66
	50% Pr S	0.578	3.48
	0% Pr S	0.802	1.80
	50% Pr C	0.456	3.86
10	0% Pr C	1.084	1.18

The results demonstrate that a) for samples with 50% deficiency of either Protein C or Protein S a higher resolution versus the normal samples and b) for samples from pregnant women a smaller deviation from normal samples is obtained with the chromogenic assay, thus supporting that a higher sensitivity and specificity will be obtained with a global chromogenic assay according to the invention as compared to a global clotting method according to the prior art.

Example 10

Effect of a mixture of Mg^{2+} and Mn^{2+} in a phospholipid reagent or in an APC reagent on discrimination for the FV:Q506 mutation in a chromogenic thrombin generation assay using Factor Xa as activator.

Sample: Plasma with normal factor V (R506R) and with hetero- (R506Q) and homozygosity (Q506Q) for FV:Q506 mutation.

Sample dilution: 1:41 in 0.05 mol/L Hepes pH 7.7, 0.15 mol/L NaCl.

Reagent A: Human prothrombin, 19 µg/mL
Phospholipids (43% phosphatidylcholine, 27% phosphatidylserine and 30% sphingomyelin), 50 µmol/L

Reagent B: Bovine Factor Xa, 0.2 nmol/L
APC, 6 µg/mL
$CaCl_2$, 25 mmol/L
Chromogenic thrombin substrate: S-2796 (Chromogenix AB), 1.8 mmol/L
Mixture of metal ions: Mg^{2+} 0.4 mmol/L, and Mn^{2+},0.04 mmol/L included in either Reagent A or Reagent B.

For carrying out the assay 50 µL of Reagent A was mixed with 50 µL of Reagent B, whereafter the mixture was incubated for three minutes at 37°C. Thereafter, 50 µL of the plasma dilution was added and incubated for two minutes at 37°C. Following that, 50 µL of the chromogenic substrate S-2796 was added and kinetic reading was performed. The change in $OD_{405-490}$ per minute was determined and expressed as $\Delta OD_{405-490}$/min.

Assay:		
	Reagent A	50 µL
	Reagent B	50 µL
	Incubate at 37°C for 3 min	
	Plasma dilution	50 µL
	2 min, 37°C	
	S-2796	50 µL
	Kinetic reading	

Results:

	Mg^{2+} and Mn^{2+} in Reagent A	Mg^{2+} and Mn^{2+} in Reagent B
FV:R506R	0.143	0.193
FV:R506Q	0.646	0.616
FV:Q506Q	1.116	0.942

The results show that a mixture of metal ions such as Mg^{2+} and Mn^{2+} may be added in a phospholipid containing reagent (Reagent A) or in a reagent containing active enzymes such as APC and Factor Xa (Reagent B)and provide a high discrimination for the FV:Q506 mutation. Hence the

addition of further metal(s) ions is not restricted to any unique reagent.

Example 11

Substitution of chloride anions with nitrate and sulfate anions in a study on the effect of magnesium and manganese in determination of Protein C activity in a three-stage thrombin generation assay.

Experimental details as in Example 1.

Magnesium nitrate ($Mg(NO_3)_2$) and manganese sulfate ($MnSO_4$) were used instead of the corresponding chloride salts at final concentrations in the assay of 0.4 and 0.04 mmol/L respectively in accordance with the conditions in Example 1. Absorbance readings are expressed as $OD_{405-490nm}$.

Results:

	Protein C, IU/mL			
	0	0.1	0.5	1.0
Ca^{2+}, 1.5 mmol/L + Mn^{2+}, 0.04 mmol/L	0.563	0.541	0.278	0.079
Ca^{2+}, 1.5 mmol/L + Mg^{2+}, 0.4 mmol/L	0.603	0.554	0.448	0.154

The results show that a similar high resolution is obtained as when using chloride as an anion (cf. Example 1). Thus, the choice of the anion is not restricted to chloride ions.

Example 12

Effect of metal ions on the detection of Protein C deficiency, Protein S deficiency and FV:Q506 in a global chromogenic method for the Protein C anticoagulant pathway, using recombinant tissue factor as activator of coagulation and monitoring thrombin generation,

Experimental details as in Example 7, but using recombinant tissue factor (PT-Fibrinogen Recombinant, Instrumentation Laboratory, Milano, Italy) instead of Thromborel as activator of coagulation. PT-Fibrinogen

Recombinant was reconstituted with 8 mL of water according to the manufacturer's instructions, thereafter diluted in 25 mmol/L Tris-HCl pH 7.6, 20 mmol/L NaCl, 0.2% bovine serum albumin to yield a final concentration during activation of coagulation of 0.25% (v/v).

Samples: Normal human plasma, Protein C deficient plasma and Protein S deficient plasma (Instrumentation Laboratory, Milano, Italy). Plasmas with 25% activity of Protein C and Protein S, respectively, were prepared by mixing normal human plasma with the Protein C or Protein S deficient plasmas respectively. Furthermore, a sample from an individual with heterozygosity for the factor V mutation (FV:R506Q) and from an individual with homozygosity for the same mutation (FV:Q506Q) were analyzed.

The analysis was performed with or without Mg^{2+} ions added to the Protein C activator solution.

Results: See Figure 5. The table below presents all primary data expressed as $OD_{405\text{-}490nm}$.

Sample	6.6 mM Ca^{2+}	6.6 mM Ca^{2+} + 0.4 mM Mg^{2+}
Normal Plasma	0.881	0.364
25% Protein C	1.225	1.047
0% Protein C	1.418	1.388
25% Protein S	1.325	0.837
0% Protein S	1.456	1.421
FV:R506Q	1.140	0.686
FV:Q506Q	1.392	1.398

The results show that the presence of magnesium ions during the Protein C activation and during the ensuing thrombin generation provides an enhancement of the anticoagulant activity (see results for normal plas-

ma). Furthermore, the enhanced anticoagulant activity results in a higher resolution at different Protein C and Protein S activity levels respectively, as well as higher discrimination for the FV:Q506 mutation.

Example 13

Effect of manganese ions on the discrimination at different Protein S activity levels in a global chromogenic method for the Protein C anticoagulant pathway, using tissue factor as activator of coagulation and monitoring thrombin generation.

Experimental details as in Example 7, using Protac® C as Protein C activator and Thromborel as activator of coagulation.

Samples: Normal human plasma (Instrumentation Laboratory, Milano, Italy) as 100% free Protein S sample; Protein S deficient plasma (Instrumentation Laboratory, Milano, Italy) as 0% free Protein S sample; plasma samples were prepared by mixing normal human plasma and Protein S deficient plasma to yield 20%, 40%, 60% and 80% free Protein S activity respectively.

The analysis was performed with or without Mn^{2+}, ions, respectively, added to the Protein C activator solution.

Results: All primary data expressed as $OD_{405-490nm}$ are listed in the table below and also illustrated in Figure 6.

Sample	6.6mM Ca^{2+}	6.6mM Ca^{2+} +0.04mM Mn^{2+}
0% Protein S	0.458	1.371
20% Protein S	0.407	0.756
40% Protein S	0.388	0.570
60% Protein S	0.367	0.493
80% Protein S	0.348	0.439

1	100% Protein S	0.325	0.379

Figure 6 shows that the addition of Mn^{2+} ions dramatically increases the resolution when compared to the use of Ca^{2+} alone, thus constituting an improved detection of Protein S deficiency in a global chromogenic method for detection of deficiency states of components in the Protein C anticoagulant pathway.

Example 14

Effect of magnesium and manganese ions on the discrimination at different Protein C activity levels in a global chromogenic method for the Protein C anticoagulant pathway, using recombinant tissue factor as activator of coagulation, a recombinant Protein C activator and monitoring thrombin generation.

Experimental details as in Example 7, but using recombinant Protein C activator as Protein C activator and recombinant tissue factor (PT-Fibrinogen Recombinant, Instrumentation Laboratory) as activator of coagulation. Recombinant Protein C activator was used as a stock solution containing 26 U/mL. The recombinant Protein C activator was then diluted in 25 mmol/L Tris-HCl pH 7.6, 20 mmol/L NaCl, 0.2% bovine serum albumin to yield a final concentration during activation of Protein C of 0.025 U/mL. PT-Fibrinogen Recombinant was prepared as in Example 12 to yield a concentration during activation of coagulation of 0.17% (v/v).

Samples: Normal human plasma (Instrumenation Laboratory, Milano, Italy) as 100% Protein C sample; Protein C deficient plasma (Instrumentation Laboratory, Milano, Italy) as 0% Protein C sample; plasma samples were prepared by mixing normal human plasma and Protein C deficient plasma to yield 20%, 40%, 60% and 80% Protein C activity respectively.

The analysis was performed with or without Mg^{2+} or Mn^{2+} ions, respectively, added to the recombinant Protein C activator solution.

Results: All primary data expressed as $OD_{405-490nm}$ are listed in the table below and also illustrated in Figure 7.

Sample	6.6mM Ca^{2+}	6.6mM Ca^{2+} + 0.4mM Mg^{2+}	6.6mM Ca^{2+} +0.4 mM Mn^{2+}
0% Protein C	1.442	1.483	1.451
20% Protein C	1.253	1.219	1.097
40% Protein C	1.010	0.900	0.636
60% Protein C	0.783	0.675	0.438
80% Protein C	0.787	0.569	0.356
100% Protein C	0.672	0.456	0.280

Figure 7 shows that the addition of Mg^{2+} or Mn^{2+} ions results in a higher resolution for the different Protein C activities when compared to the use of Ca^{2+} alone, thus resulting in an improved detection of Protein C deficiency in a global chromogenic method for detection of deficiency states of components in the Protein C anticoagulant pathway. The results further illustrate the applicability of recombinant sources of tissue factor and Protein C activator.

Example 15

Effect of the combination of Mg^{2+} and Mn^{2+} ions on the detection of Protein C deficiency, Protein S deficiency and FV:Q506 in a global chromogenic method for the Protein C anticoagulant pathway, using tissue factor as activator of coagulation and monitoring thrombin generation.

Expermimental details as in Example 7, using Protac® C as Protein C activator and Thromborel as activator of coagulation.

Samples: Normal human plasma, Protein C deficient plasma and Protein S deficient plasma (Instrumentation Laboratory, Milano, Italy). Further-

more, a sample from an individual with heterozygosity for the factor V mutation(FV:R506Q) and from an individual with homozygosity for the same mutation (FV:Q506Q) were analyzed

The analysis was performed with or without the presence of the combination of Mg^{2+} and Mn^{2+} ions added to the Protein C activator solution.

Results: See Figure 8. The table below presents all primary data expressed as ΔAbnormal - Normal (i.e. $DO_{405-490}$ abnormal plasma - $DO_{405-490}$ normal plasma).

Sample	6.6 mM Ca2+	6.6 mM Ca2+ + 0.4 mM Mg2+ + 0.04 mM Mn2+
0% Protein C	0.425	0.801
0% Protein S	0.372	0.742
FV:R506Q	0.060	0.413
FV:Q506Q	0.530	0.664

The results show that the addition of the combination of magnesium and manganese ions to calcium ions provide a higher resolution for both Protein C and Protein S deficiencies, as well as a higher discrimination for the FV:Q506 mutation, thus proving the beneficial use of adding a combination of metal ions in a global chromogenic method.

Claims

1. In vitro photometric method for qualitative screening and quantitative determination of the functional activity of components of the Protein C anticoagulant pathway of blood coagulation, comprising measuring the conversion rate of an exogenous substrate by an enzyme, the activity of which is related to the Protein C anticoagulant activity, in a blood sample of a human comprising coagulation factors and said exogenous substrate after at least partial activation of coagulation through the intrinsic, extrinsic or common pathway and triggering coagulation by adding calcium ions; and comparing said conversion rate with the conversion rate of a normal human blood sample determined in the same way, **characterized by** adding further metal(s) ions selected from divalent metal ions and monovalent copper ions to said sample.

2. The method according to claim 1, **characterized by** using Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Sr^{2+}, and/or Cu^{+} ions as said further metal(s) ions.

3. The method according to claims 1 and 2 for the global screening for defects in the Protein C anticoagulant pathway of blood coagulation in a human, comprising:

A) incubating a blood sample of said human comprising coagulation factors with:

1) an activator for the Protein C in said sample,

2) a suitable coagulation activator,

3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

4) calcium ions, and

5) said further metal(s) ions;

B) determining the conversion rate of said exogenous substrate; and

C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

4. The method according to claims 1 and 2 for the determination of free Protein S activity in a blood sample of said human, comprising:

A) incubating said blood sample comprising coagulation factors with:
1) exogenous activated Protein C or exogenous Protein C together with an activator of Protein C,
2) a suitable coagulation activator,
3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,
4) calcium ions, and
5) said further metal(s) ions;
B) determining the conversion rate of said exogenous substrate; and
C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

5. The method according to claims 1 and 2 for the determination of Protein C activity in a blood sample of said human, comprising:
A) incubating a blood sample of said human comprising coagulation factors with:
1) an activator for the Protein C in said sample,
2) a suitable coagulation activator,
3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,
4) calcium ions, and
5) said further metal(s) ions;
B) determining the conversion rate of said exogenous substrate; and
C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

6. The method according to claims 1 and 2 for screening for Factor V mutation(s) in a blood sample of said human, comprising:
A) incubating a blood sample of said human comprising coagulation factors with:
1) exogenous activated Protein C, or exogenous Protein C together with an activator of Protein C, or an activator for endogenous Protein C,
2) a suitable coagulation activator,
3) an exogenous synthetic substrate for either Factor Xa or thrombin com-

prising a photometrically measurable leaving group,

4) calcium ions, and

5) said further metal(s) ions;

B) determining the conversion rate of said exogenous substrate; and

C) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

7. The method according to any one of the preceding claims, **characterized in that** the blood sample is blood or a blood derived sample such as a blood plasma sample or a blood serum sample.

8. The method according to any one of claims 4 to 7, **characterized in that** said stages 1) to 5) of incubation step A, i.e. 1) addition of an activator for endogenous Protein C, or of exogenous activated Protein C, or of exogenous Protein C together with an activator of Protein C, 2) addition of a suitable coagulation activator to provide at least partial activation of coagulation, 3) addition of an exogenous synthetic substrate for either Factor Xa or thrombin 4) addition of calcium ions, and 5) addition of said further metal(s) ions respectively, can be performed separately and/or simultaneously.

9. The method according to any one of the preceding claims, **characterized by** adding said further metal(s) ions in the Protein C activation stage .

10. The method according to any one of the preceding claims, **characterized in that** said calcium ions are used in a concentration of 0.5 to 20 mmol/L, preferably 1 to 10 mmol/L of the final assay medium.

11. The method according to any one of the preceding claims, **characterized in that** said Mg^{2+} ions are used in a concentration of 20 µmol/L to 10 mmol/L, preferably 100 µmol/L to 2 mmol/L and, more preferably 200 µmol/L to 1 mmol/L of the final assay medium.

12. The method according to any one of the preceding claims, **character-**

ized in that said Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Sr^{2+}, and/or Cu^{+} ions are used in a concentration of 1 µmol/L - 2mmol/L, preferably 5 to 400 µmol/L and, more preferably 10 to 80 µmol/L of the final assay medium.

13. The method according to any one of the preceding claims, **characterized in that** the activation of Protein C in said sample precedes or occurs simultaneously with activation of coagulation.

14. The method according to any one of the preceding claims, **characterized in that** an activator for Protein C selected from the group comprising Protein C activating snake venom enzymes and thrombin, if desired thrombin in combination with thrombomodulin, is used.

15. The method according to any one of the preceding claims, **characterized in that** a recombinant activator for Protein C is used.

16. The method according to claim 14, **characterized in that** a Protein C activating snake venom enzyme obtained from the Agkistrodon family of snakes, preferably from *Agkistrodon contortrix contortrix* is used.

17. The method according to claim 16, **characterized in that** the crude venom or the snake venom enzyme preparation Protac® C is used.

18. The method according to claim 17, **characterized by** using the Protein C activator Protac® C in an amount of $1x10^{-3}$ to 1 U/mL, preferably $2x10^{-3}$ to 0.3 U/mL in the final assay medium.

19. The method according to any one of the preceding claims, **characterized by** using as suitable coagulation activators for the intrinsic pathway compositions comprising

a) phospholipid(s) and

b) contact activators and/or activated Factors IX, XII or XI or a reagent which generates activated factors IX, XII or XI in vitro,

20. The method according to claims 18 and 19, **characterized by** using a contact activator selected from the group comprising ellagic acid, collagen, collagen related substances or a silica, such as micronized silica, colloidal silica and kaolin.

21. The method according to any one of the preceding claims, **characterized by** using native or recombinant human or non-human tissue factor (thromboplastin) from human or non-human species with or without Factor VII/VIIa, or (native or recombinant human or non-human) Factor VIIa and phospholipids as a suitable coagulation activator for the extrinsic pathway.

22. The method according to any one of claims 1, 3 to 6, 8, 19, 21 and 22, **characterized by** using in the at least partial activation of coagulation according to the intrinsic, extrinsic or common pathway phospholipid(s) selected from synthetic phospholipids, purified phospholipids or a mixture thereof, or crude extracts of biological sources, specifically extracts from brain, platelets, placenta, egg yolk or from soybeans.

23. The method according to any one of the preceding claims, **characterized by** using exogenous activated Factor X, or exogenous Factor X and an exogenous activator for Factor X, or an exogenous activator for endogenous Factor X as a suitable coagulation activator for the common pathway.

24. The method according to claim 23, **characterized by** using a snake venom enzyme from Russelli Viperii as an exogenous activator for Factor X.

25. The method according to any one of the preceding claims, **characterized in that** components of the Protein C anticoagulant pathway are added to the reaction medium to compensate for variable functional levels of such components in the sample.

26. The method according to claim 25, **characterized by** using compo-

nents of the Protein C anticoagulant pathway selected from the group comprising Protein C, activated Protein C, Protein S, Factor V/Factor Va, or a plasma deficient of the actual Protein C anticoagulant pathway component to be measured or a plasma deficient of all said components of the Protein C anticoagulant pathway.

27. The method according to any one of the preceding claims, **characterized in that** a fibrin polymerization inhibitor, such as Gly-Pro-Arg-Pro is added to the reaction medium.

28. The method according to any one of the preceding claims, **characterized in that** coagulation factors selected from the group comprising Factor VIII/Factor VIIIa, Factor IX, Factor X and prothrombin are added to the reaction medium.

29. The method according to any one of the preceding claims, **characterized in that** the coagulation factors used are selected from human or non-human sources or being produced by recombinant technology as wild-type proteins or as modified polypeptides to provide the suitable functional property.

30. The method according to any one of the preceding claims, **characterized by** using an exogenous synthetic substrate for either Factor Xa or thrombin in the reaction mixture.

31. The method according to claim 30, **characterized in that** as the exogenous synthetic substrate for either Factor Xa or thrombin a photometric substrate comprising a chromophore, a fluorophore or a luminophore as a leaving group is used.

32. The method according to claim 31, **characterized in that** a photometric substrate comprising a p-nitroaniline group (pNA) as a chromophoric leaving group, a naphthylamine or coumarine derivative group as a fluorophoric leaving group, and an isoluminolamide group as a lumino-

phoric leaving group.

33. The method according to claim 30 to 32, **characterized in that** as substrate for Factor Xa Benzoyl-Ile-Glu-Gly-Arg-pNA (S-2222), N-a-Z-D-Arg-Gly-Arg-pNA (S-2765), CH_3SO_2-D-Leu-Gly-Arg-pNA (CBS 31.39) or MeO-CO-D-CHG-Gly-Arg-pNA (Spectrozyme Xa) is used.

34. The method according to claim 30 to 32, **characterized in that** as a substrate for thrombin H-D-Phe-Pip-Arg-pNA (S-2238), pyroGlu-Pro-Arg-pNA (S-2366), H-D-Ala-Pro-Arg-pNA (S-2846), Z-D-Arg-Sarc-Arg-pNA (S-2796), AcOH*H-D-CHG-But-Arg-pNA CBS 34.47) or H-D-HHT-Ala-Arg-pNA (Spectrozyme TH) is used.

35. A kit for use in the methods according to any one of the preceding claims comprising the following components:
a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;
b) a suitable coagulation activator;
c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;
d) calcium ions; and
e) said further metal(s) ions;
in separate containers and/or in containers comprising mixtures of at least two of said components in aqueous solution or in lyophilized form. .

36. A kit for use in the methods according to any one of the preceding claims comprising the following components:
a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;
b) a suitable coagulation activator;
c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;
d) calcium ions;
e) said further metal(s) ions; and

f) coagulation factors;

in separate containers and/or in containers comprising mixtures of at least two of said components in aqueous solution or in lyophilized form. .

37. A reagent for use in the methods according to any one of claims 1 to 34, **characterized by** comprising said further metal(s) ions and at least one of the following components a) to e):

a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;

b) a suitable coagulation activator;

c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;

d) calcium ions; and

e) coagulation factors;

in one container in aqueous solution or in lyophilized form.

38. The reagent according to claim 37, **characterized by** comprising at least two of said components a) to e) and said further metal(s) ions in one container in aqueous solution or in lyophilized form.

39. The reagent according to claim 38, **characterized by** comprising activated Protein C, calcium ions and said further metal(s) ions in one container in aqueous solution or in lyophilized form.

40. The reagent according to claim 38, **characterized by** comprising activated Protein C, and said further metal(s) ions in one container in aqueous solution or in lyophilized form.

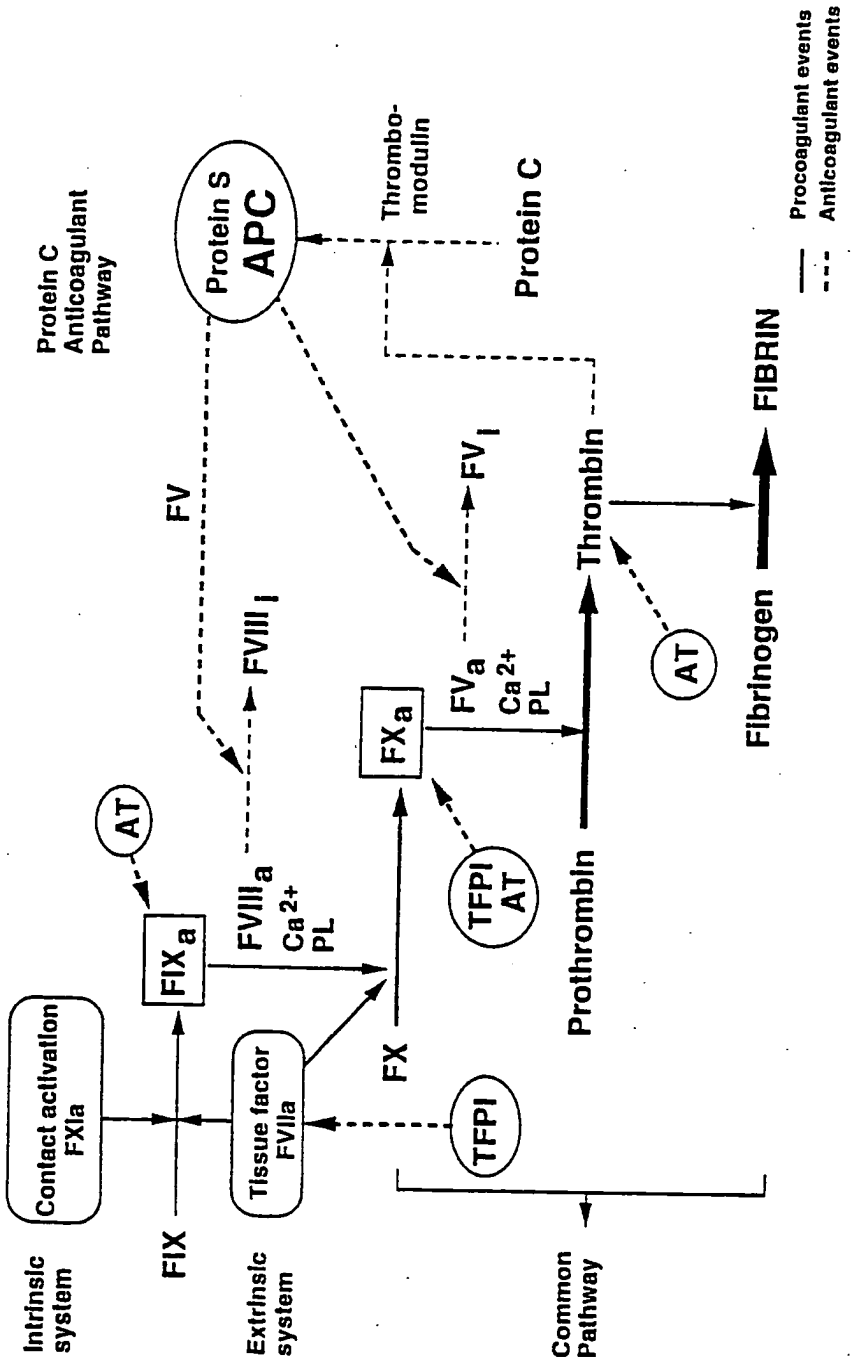
41. The reagent according to claim 37, **characterized by** comprising coagulation factors and said further metal(s) ions in one container in aqueous solution or in lyophilized form.

42. The reagent according to claim 41, **characterized by** additionally containing phospholipid(s).

43. The reagent according to claim 37 and 42, **characterized by** comprising said further metal(s) ions in combination with one or more of Factor V/Va, Protein C, Protein S, prothrombin, Factor VIII/VIIIa, Factor IX/IXa, Factor X/Xa, and/or thrombin in one container in aqueous solution or in lyophilized form.

44. The reagent according to claim 37, **characterized by** comprising said further metal(s) ions in combination with a Protein C activator, such as Protac®C or thrombin/thrombomodulin, in one container in aqueous solution or in lyophilized form.

Fig. 1


Intrinsic system
Extrinsic system
Common Pathway
Contact activation FXIa
Tissue factor FVIIa
FIX
FIXa
AT
FVIIIa
Ca2+
PL
FVIII i
FV
Protein C Anticoagulant Pathway
Protein S APC
Thrombo-modulin
Protein C
FX
FXa
TFPI
TFPI AT
FVa
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FV i
Prothrombin
Thrombin
AT
Fibrinogen
FIBRIN
Procoagulant events
Anticoagulant events

Figure 2

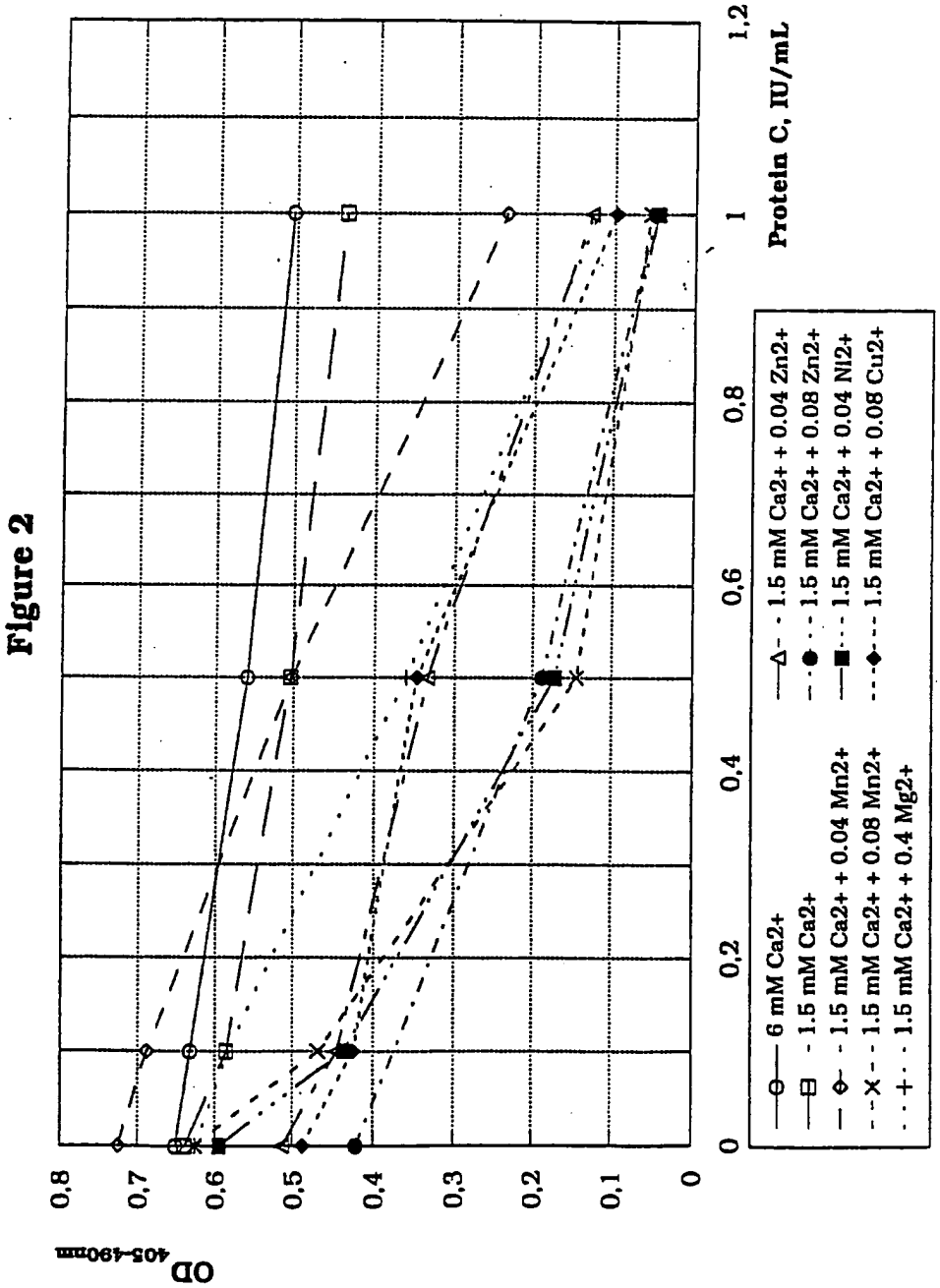

OD405-490nm
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
0,2
0,4
0,6
0,8
1
1,2
Protein C, IU/mL
6 mM Ca2+
1.5 mM Ca2+
1.5 mM Ca2+ + 0.04 Mn2+
1.5 mM Ca2+ + 0.08 Mn2+
1.5 mM Ca2+ + 0.4 Mg2+
1.5 mM Ca2+ + 0.04 Zn2+
1.5 mM Ca2+ + 0.08 Zn2+
1.5 mM Ca2+ + 0.04 Ni2+
1.5 mM Ca2+ + 0.08 Cu2+

Figure 3

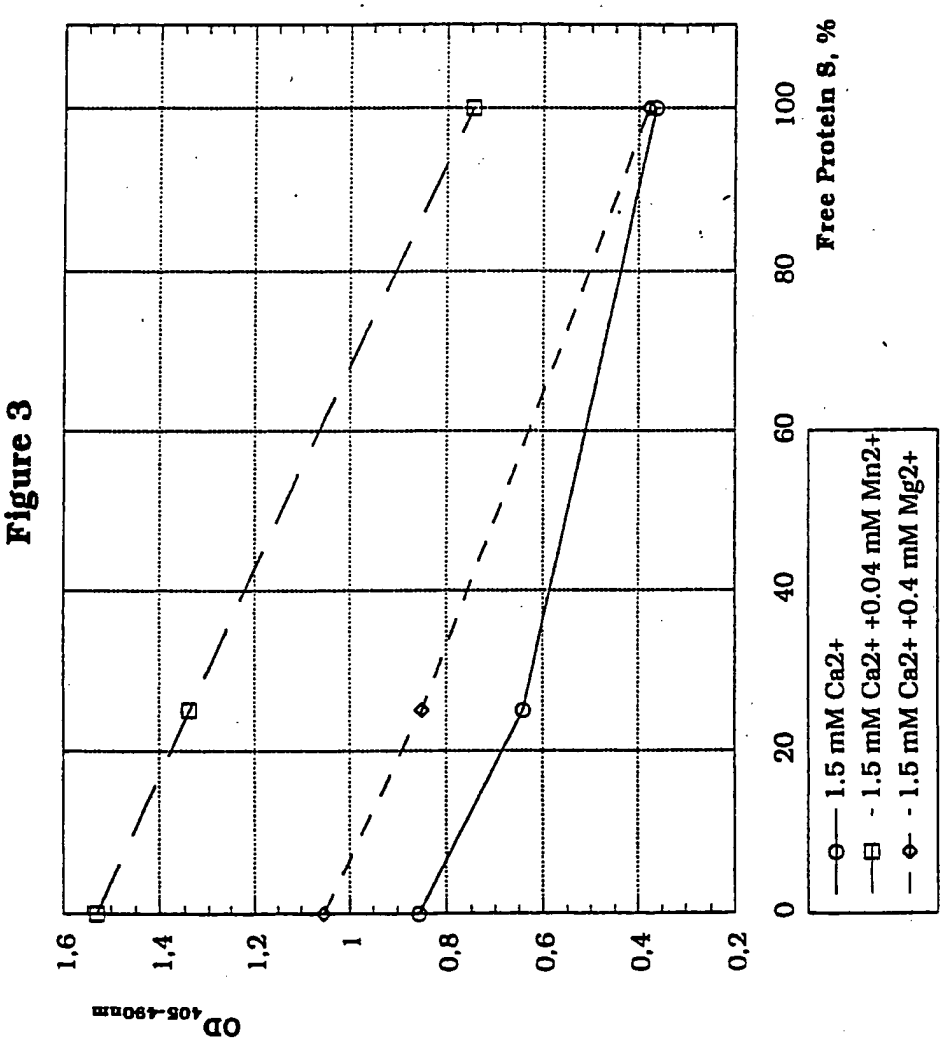

OD405-490nm
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
Free Protein S, %
0
20
40
60
80
100
1.5 mM Ca2+
1.5 mM Ca2+ +0.04 mM Mn2+
1.5 mM Ca2+ +0.4 mM Mg2+

Figure 4

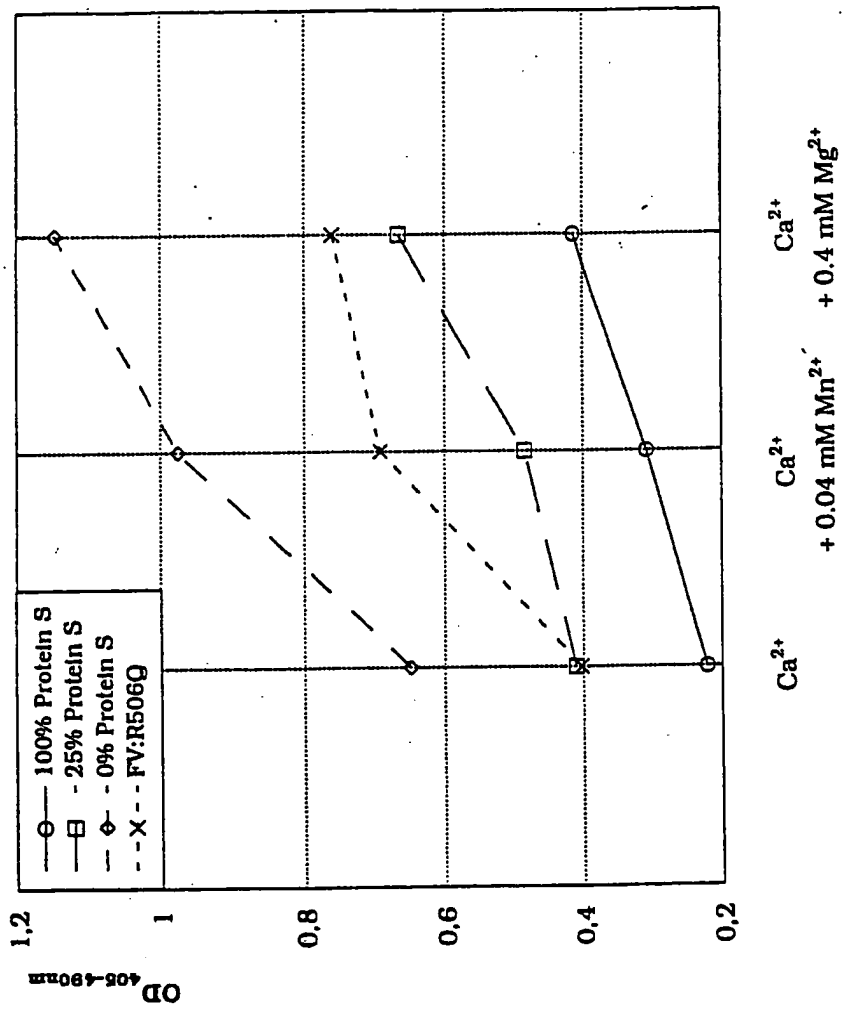

100% Protein S
- 25% Protein S
- 0% Protein S
- FV:R506Q
OD405-490nm
1,2
1
0,8
0,6
0,4
0,2
Ca2+
Ca2+
+ 0.04 mM Mn2+
Ca2+
+ 0.4 mM Mg2+

Figure 5

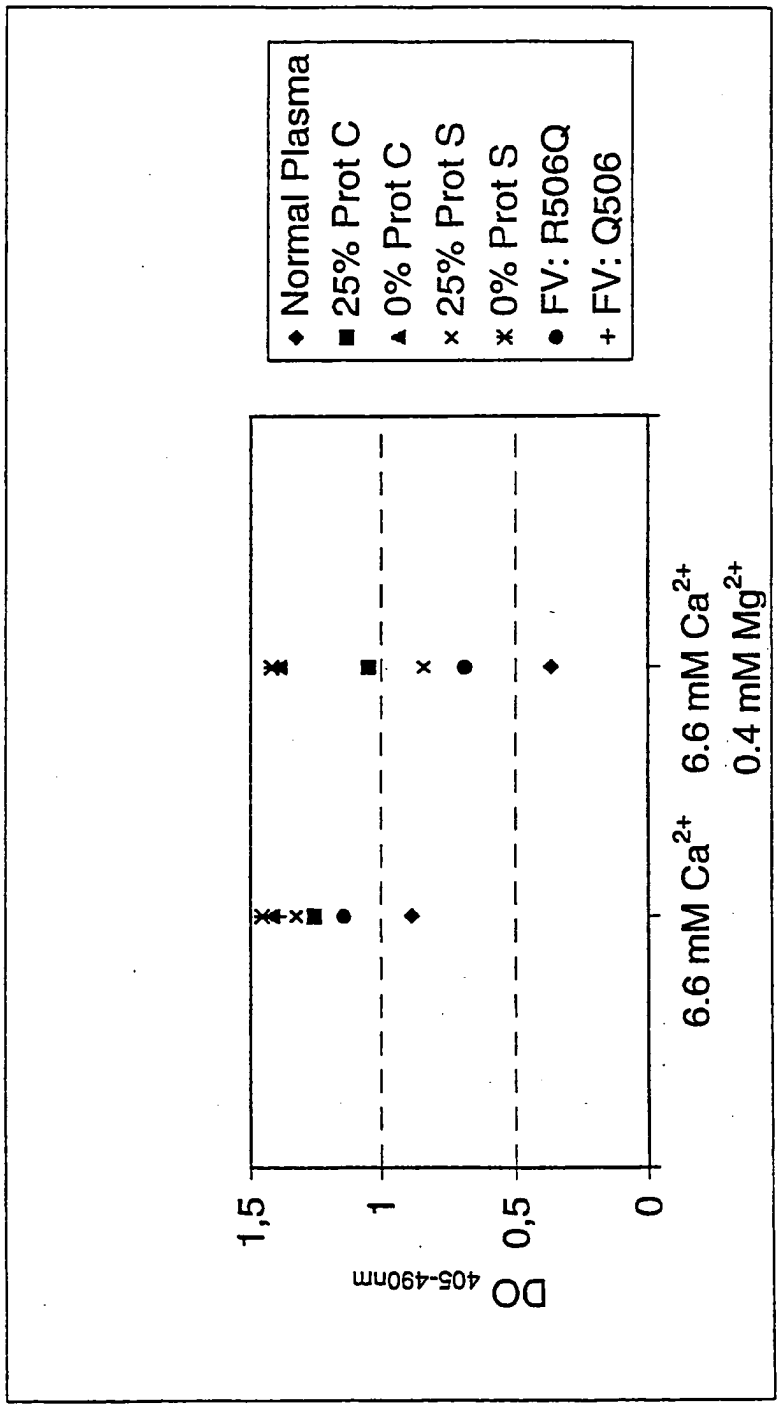
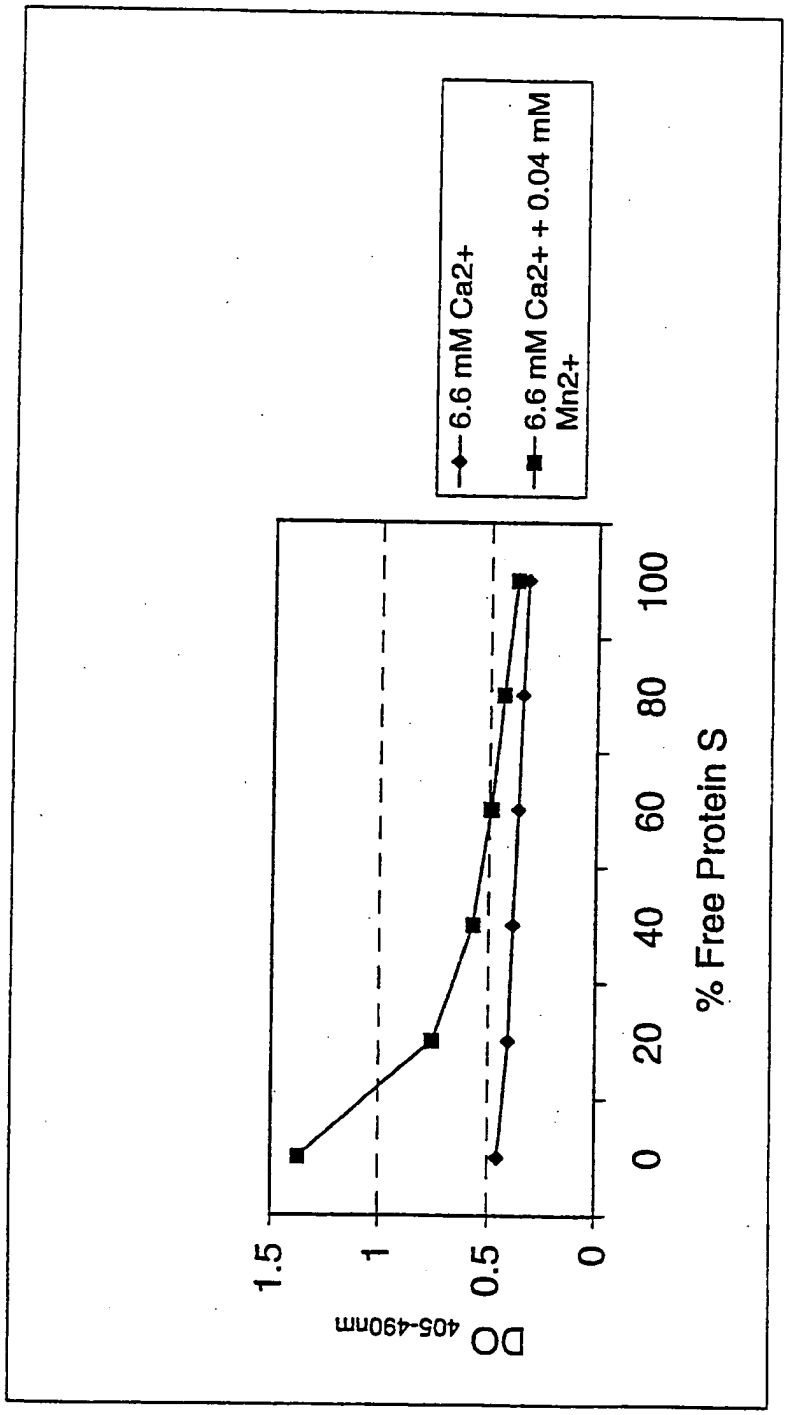

Normal Plasma
25% Prot C
0% Prot C
25% Prot S
0% Prot S
FV: R506Q
FV: Q506
DO 405-490nm
1,5
1
0,5
0
6.6 mM Ca2+
6.6 mM Ca2+
0.4 mM Mg2+

Figure 6


6.6 mM Ca2+
6.6 mM Ca2+ + 0.04 mM Mn2+
DO 405-490nm
1.5
1
0.5
0
0
20
40
60
80
100
% Free Protein S

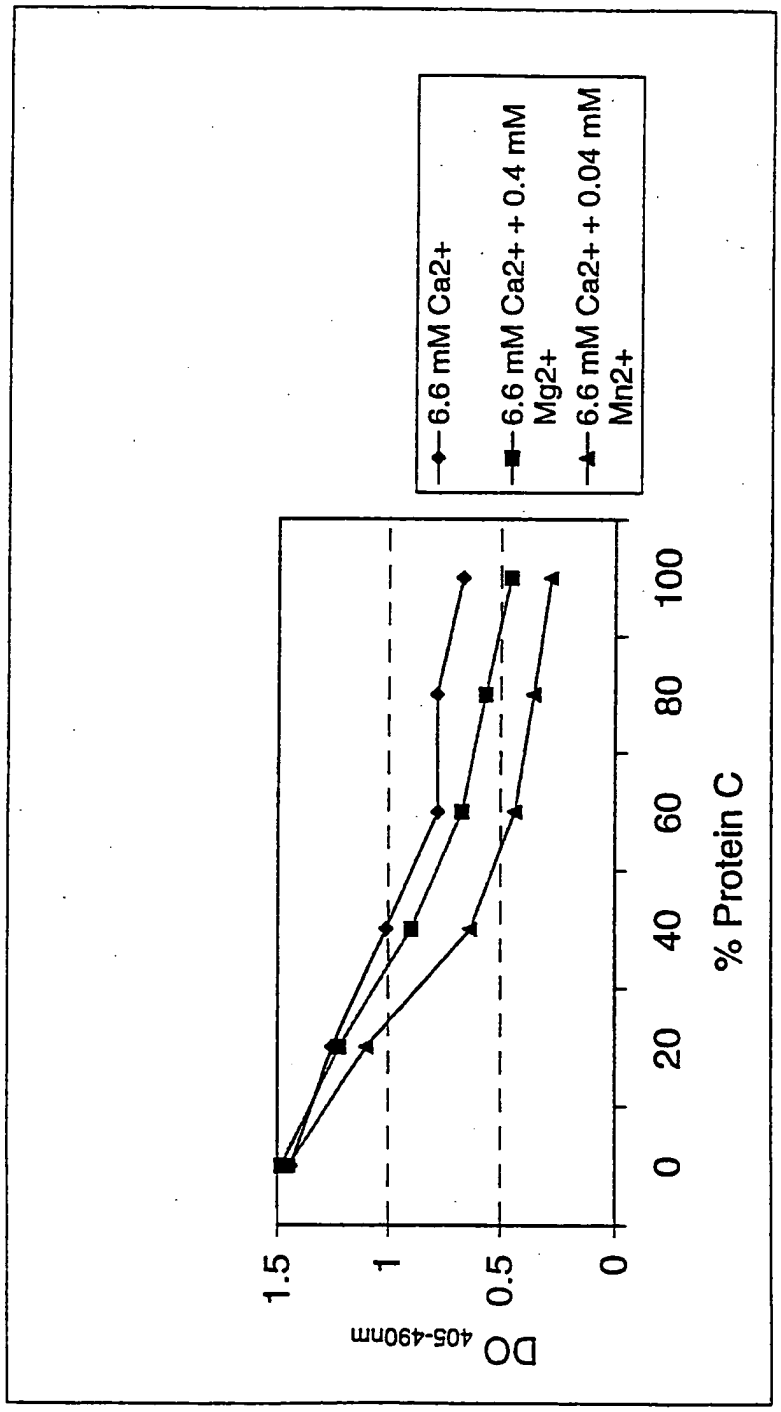

6.6 mM Ca2+
6.6 mM Ca2+ + 0.4 mM Mg2+
6.6 mM Ca2+ + 0.04 mM Mn2+
DO 405-490nm
1.5
1
0.5
0
0
20
40
60
80
100
% Protein C

Figure 7

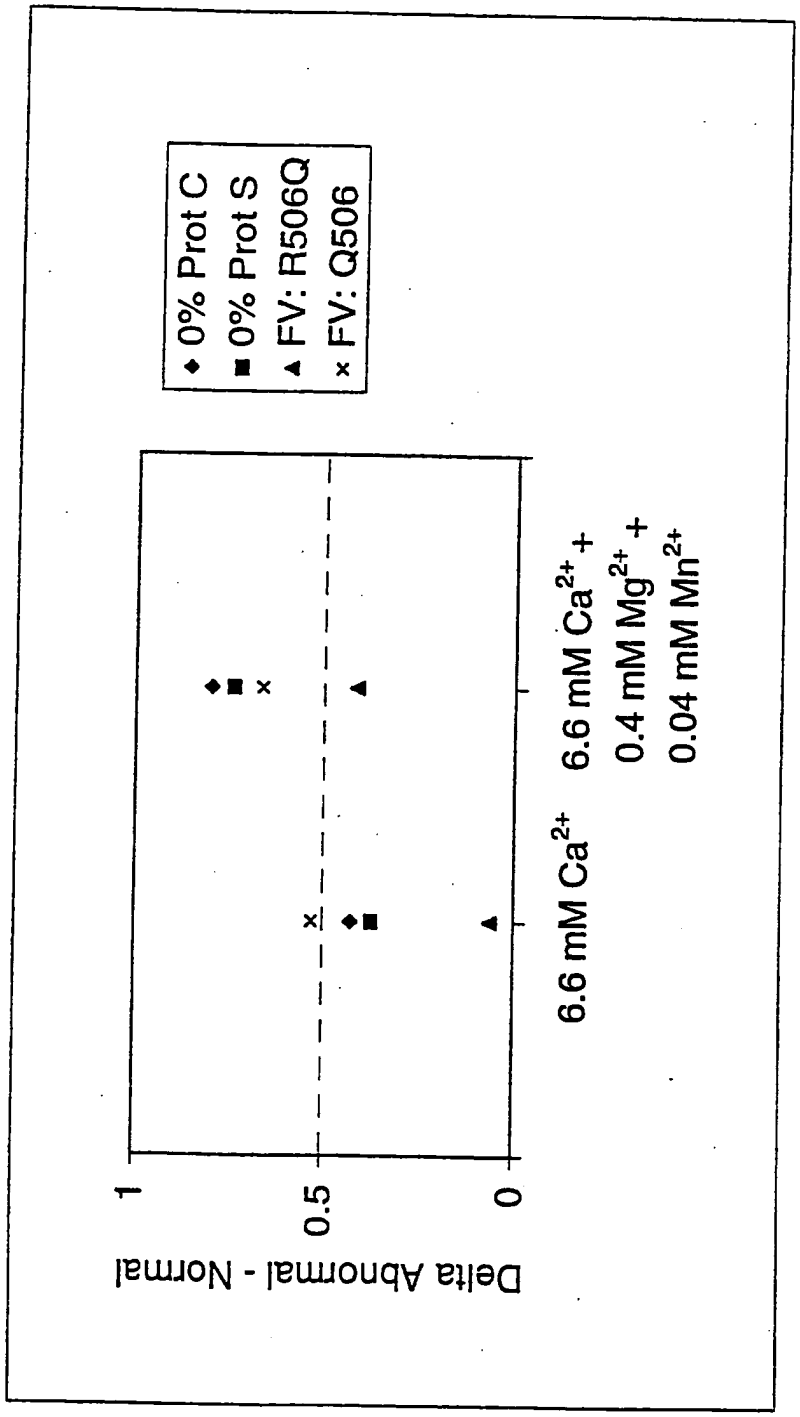

◆ 0% Prot C
■ 0% Prot S
▲ FV: R506Q
× FV: Q506
Delta Abnormal - Normal
1
0.5
0
6.6 mM Ca2+
6.6 mM Ca2+ +
0.4 mM Mg2+ +
0.04 mM Mn2+

Figure 8

INTERNATIONAL SEARCH REPORT

Interna ..al Application No
PCT/EP 99/01599

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/56 G01N33/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93 10262 A (BAXTER DIAGNOSTICS INC) 27 May 1993 (1993-05-27) the whole document & EP 0 567 636 A (BAXTER DIAGNOSTICS INC) 3 November 1993 (1993-11-03) cited in the application the whole document	1-44
Y	US 5 001 069 A (BARTL KNUT ET AL) 19 March 1991 (1991-03-19) cited in the application the whole document	1-44

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1999	27/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hart-Davis, J

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INTERNATIONAL SEARCH REPORT

Internat. al Application No
PCT/EP 99/01599

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 5 055 412 A (PROKSCH GARY J) 8 October 1991 (1991-10-08) the whole document ---	1-44
Y	F.SEKIYA, T.YAMASHITA, H.ATODA, Y.KOMIYAMA, T.MORITA: "Regulation of the Tertiary Structure and Function of Coagulation Factor IX by Magnesium(II) Ions" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 24, 16 June 1995 (1995-06-16), pages 14325-14331, XP002067666 cited in the application the whole document ---	1-44
A	M.J.HEEB, A.GRUBER, J.H.GRIFFIN: "Identification of Divalent Metal Ion-dependent Inhibition of Activated Protein C by alpha2-Macroglobulin and alpha2-Antiplasmin in Blood and Comparisons to Inhibition of Factor Xa, Thrombin, and Plasmin" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 26, 15 September 1991 (1991-09-15), pages 17606-17612, XP002067667 cited in the application the whole document ---	1-44

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Internat. al Application No
PCT/EP 99/01599

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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PCT/EP 99/01599

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